Università degli Studi di Roma "TOR VERGATA"

Faculty of Medicine and Surgery
Faculty of Science

Doctoral thesis in Immunology and Applied Biotechnology

XXII Cycle

Immunological aspects of paediatric patients with primary immunodeficiency

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A.A. 2009/2010

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Ma è una bottega
lo spazio della vita
Una bottega aperta
all'inverno pungente
all'estenuazione estiva
alla fragilità di primavera
e alla serena invasione
delle foglie d'autunno…

Vittorio Ferrero

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ABSTRACT

DiGeorge syndrome (DGS) is caused by a deletion in hemizygosis of 22q11.2 locus responsible for embryogenesis defects causing alterations of thymus and parathyroid glands, cardiac defects and abnormal facial features. In most patients, the immune defect is basically in T cell subset although abnormalities such as dysgammaglobulinemia, IgA and memory B cells deficiency have been also reported.

On the basis of the immunologic findings, DGS patients are divided in complete DGS (cDGS), a rare form of severe combined immune deficiency (0.5-1%), and partial DGS (pDGS) presenting mild/moderate T cell lymphopenia. Clinically, these patients display a wide spectrum of infections together with a dysregulation of immune system as atopic and autoimmune manifestations. T cell levels, although reduced, are not predictive of the risk to develop infections or autoimmunity.

The alterations of the T cell receptor (TCR) repertoire distribution and impaired thymic output in pDGS have been variably associated to a higher risk of infections or autoimmunity.

In order to better define some aspects of the pathogenesis and immunological features we studied the kinetic of immune reconstitution in a cohort of pDGS patients and in patients affected by other primary immunodeficiencies not directly affecting T-cell

compartments (as Chronic Granulomatous Disease patients) through peripheral blood mononuclear cells (PBMC) analysis of:

1. Phenotype and immunologic functions through standard techniques.
2. TCR repertoire distribution of T $CD4^+$ and T $CD8^+$ subsets (TCRVB spectratyping).
3. Immunophenotypic B-cell maturation.
4. Frequency of T regulatory cells.

The correlation between the *in vitro* immunological profile and the clinical features might help to clarify some aspects of the pathogenesis of the immunological defects, in order to identify possible prognostic markers of increased risk of susceptibility to infections or of development of autoimmunity.

Results and Discussion: pDGS patients exhibited decreased T-cell numbers, although no correlation was found between low T-cell values and recurrent infections. Total B-cell numbers in pDGS and CGD patients were normal, although a significantly decreased proportion of memory B cells was observed. No difference in natural T regulatory cells frequency was evident when compared with healthy controls in any groups.

A statistical reduced lymphoproliferative response to stimuli (PHA, OKT3 and PWM) in all patients was observed.

TCRBV family distribution resulted perturbed, with higher degree in $CD8^+$ T-cell subset in both CGD and pDGS patients. Particularly, TCRBV family alterations in pDGS patients showed a trend of

normalization in T cell repertoire distribution (both $CD4^+$ and $CD8^+$ T-cell), as observed in CMV congenital infected patients. Recurrent infections correlated with a high frequency of TCRBV family alterations in pDGS but not in CGD patients.

Although low T-cell values were not predictive of recurrent infections in DiGeorge syndrome, higher TCRBV family alterations, as well as humoral immunodeficiency and reduced $CD27^+$ B cell memory frequency, were associated to an increased risk of infections in these patients.

Interestingly analysis of CGD patients showed significative alterations in both T and B cells compartments, suggesting that quantitative and qualitative alterations found, might contribute to the heterogeneity in the clinical phenotype.

Further studies are needed to elucidate how NADPH oxidase system is involved in CGD patients immune alterations.

In conclusion, our analysis on PID patients with well known defective cell compartments revealed that other subsets were also involved, therefore some parameters such as TCRBV family distribution and B-cell maturation could be used as further prognostic markers for follow-up and specific treatment.

Key words: 22q11.2 microdeletion, Chronic Granulomatous Disease, DiGeorge Syndrome, Immune reconstitution, Primary Immunodeficiency Disease, Spectratyping, T cell receptor, T lymphocytes.

RIASSUNTO

La sindrome di DiGeorge (DGS) è causata da una delezione in emizigosi del locus 22q11.2 responsabile di difetti embriogenetici che determinano l'alterazione del timo e delle ghiandole paratiroidee, difetti cardiaci e anomalie facciali caratteristiche. In molti pazienti affetti da questa sindrome, il difetto immunitario è principalmente del compartimento T, sebbene siano state riportate anche anomalie quali disgammaglobulinemia, deficit di IgA e di cellule B della memoria. Sulla base dei dati immunologici, i pazienti DGS sono classificati in DGS completi (cDGS) e DGS parziali (pDGS): i cDGS corrispondono ad una rara forma di immunodeficienza grave combinata (0,5-1%), mentre i pDGS presentano una forma variabile di linfopenia T (da leggera a moderata). Dal punto di vista clinico, questi pazienti mostrano un ampio spettro d'infezioni insieme ad una disregolazione del sistema immunitario con manifestazioni atopiche e autoimmunitarie. I livelli cellulari T, sebbene ridotti, non sono predittivi del rischio di infezioni o autoimmunità.

Le alterazioni della distribuzione del repertorio del recettore delle cellule T (TCR) e il ridotto apporto timico nei pazienti pDGS è stato variabilmente associato ad un maggior rischio di infezioni o autoimmunità.

Per meglio definire alcuni aspetti della patogenesi e delle caratteristiche immunologiche, abbiamo studiato la cinetica della

ricostituzione immunologica in una coorte di pazienti pDGS e in pazienti affetti da altre immunodeficienze primitive non direttamente riconducibili ad alterazioni del compartimento di cellule T (come pazienti con Malattia Granulomatosa Cronica - CGD). A tal fine sono state analizzate le cellule mononucleate del sangue periferico valutando:

1. Il fenotipo e le funzioni immunologiche tramite tecniche convenzionali.
2. La distribuzione del repertorio del TCR dei compartimenti cellulari T $CD4^+$ e T $CD8^+$ (TCRBV spectratyping).
3. L'immunofenotipo di maturazione delle cellule B.
4. La frequenza delle cellule nTreg.

La correlazione tra il profilo immunologico *in vitro* e le caratteristiche cliniche può aiutare a chiarire alcuni aspetti della patogenesi e del difetto immunologico, al fine di identificare dei possibili indicatori di suscettibilità verso le infezioni o verso lo sviluppo di autoimmunità.

Risultati e Discussione: I pazienti pDGS mostravano un ridotto numero di cellule T, sebbene non sia stata trovata una correlazione tra i bassi valori di cellule T e le infezioni ricorrenti. Il numero totale di cellule B nei pazienti pDGS e CGD era normale, sebbene sia stato osservato un significativo decremento di cellule B della memoria. La frequenza delle cellule T regolatorie naturali dei pazienti non mostrava differenze se confrontata con i valori dei controlli sani di pari età.

La risposta linfoproliferativa verso ogni stimolo era statisticamente diminuita in tutti i pazienti.

La distribuzione delle famiglie dei TCRBV è risultata perturbata, con maggiore rilevanza nelle cellule T $CD8^+$ sia nei pazienti pDGS che in quelli CGD. In particolare, l'alterazione delle famiglie TCRBV ha mostrato una tendenza di normalizzazione nella distribuzione del repertorio delle cellule T (sia $CD4^+$ che $CD8^+$), come osservato anche in pazienti infetti da CMV. Le infezioni ricorrenti correlavano con una maggiore frequenza di alterazioni delle famiglie TCRBV nei pazienti pDGS ma non nei pazienti CGD.

Sebbene nella sindrome DiGeorge i bassi valori di cellule T non siano predittivi delle infezioni, le maggiori alterazioni delle famiglie TCRBV, così come l'immunodeficienza umorale e la ridotta frequenza di cellule B della memoria $CD27^+$, risultavano essere associati a un maggiore rischio di infezioni in questi pazienti.

L'analisi dei pazienti CGD ha mostrato delle alterazioni significative sia nel compartimento cellulare T che in quello B, suggerendo che le alterazioni quantitative e qualitative trovate, possono contribuire alla eterogeneità del fenotipo clinico.

Ulteriori studi sono necessari per delucidare come il sistema della NADPH ossidasi è coinvolto nell'alterazione immunologica dei pazienti CGD.

In conclusione, le nostre analisi nelle PID hanno rilevato che altri compartimenti cellulari, oltre a quelli noti, possono essere coinvolti in queste malattie. Pertanto alcuni parametri come la distribuzione

delle famiglie del TCRBV e la valutazione della maturazione delle cellule B dovrebbero essere usati come indicatori prognostici del rischio d'infezioni nel corso del follow-up per intraprendere eventuali terapie specifiche.

Parole chiave: Immunodeficienza Primitiva (PID), Malattia Granulomatosa Cronica, Recettore delle cellule T, Ricostituzione Immunologica, Sindrome di DiGeorge, Spectratyping.

LIST OF PUBBLICATIONS

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ABBREVIATIONS

APC:	Allophycocyanin dye
APC:	Antigen Presenting Cell
BCR:	B Cell Receptor
BTK:	Bruton's Tyrosine Kinase
CD40L:	CD40 Ligand
CDR:	Complementary Determining Region
CGD:	Chronic Granulomatous Disease
CMV:	Cytomegalovirus
cpm:	Counts Per Minute
cTEC:	Cortical Thymic Epithelial Cell
CTL:	Cytotoxic T lymphocyte
CVID:	Common Variable Immunodeficiency
DC:	Dendritic cell
DGS:	DiGeorge Syndrome
DN:	Double negative
DP:	Double positive
ESID:	European Society for Immunodeficiencies
FasL:	Fas ligand
FISH:	Fluorescence In Situ Hybridization
FITC:	Fluorescein isothiocyanate
GC:	Germinal Centre
HAART:	Highly Active Antiretroviral Therapy
HIV:	Human Immunodeficiency Virus
IFN-:	Interferon
Ig:	Immunoglobulin
IL-:	Interleukin
MHC:	Major histocompatibility complex
mTEC:	Medullar Thymic Epithelial Cell
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NK:	Natural killer
PBMC:	Peripheral Blood Mononuclear Cell
PE:	R-Phycoerythrin
PECy7:	R-Phycoerythrin Cy7
PHA:	Phytohaemaglutinin
PID:	Primary Immunodeficiency Disease

PWM:	Pokeweed Mitogen
RAG:	Recombination Activating Gene
ROS:	Reactive Oxygens Species
RT-PCR:	Reverse Transcriptase Polymerase Chain Reaction
SD:	Standard Deviation
SI:	Stimulation Index
SP:	Single Positive
TCR:	T Cell Receptor
Th:	T Helper
TN:	Triple Negative
TNF-:	Tumour Necrosis Factor
Treg:	Regulatory T-cell
XLA:	X-linked Agammaglobulinaemia

INTRODUCTION

THYMUS

The thymus is a complex primary lymphoid organ responsible for T cell development and central self-tolerance. Thymic compartments can be broadly divided into two main anatomical regions, the outer cortex and the inner medulla. This microenvironment that provides the unique combination of cellular interactions, cytokines, and chemokines to induce thymocytes precursors to undergo a differentiation program that leads to the generation of functional T cells.

Normal Anatomy

The mammalian thymus is located in the pericardial mediastinum. The thymus consists of two distinct lobes connected by a connective tissue isthmus.

The thymic arteries enter the organ substance at the corticomedullary junction ramify into capillaries that extend into the cortex and medulla. Capillaries in the cortex are rarely fenestrated. This restricts access of circulating antigenic molecules to developing cortical lymphocytes. By contrast, medullary capillaries are fenestrated and freely permeable to circulating antigens. Prothymocytes are thought to enter the thymic stroma through the large venules at the corticomedullary junction (CMJ), and re-enter the circulation through the vascular lining of post-capillary venules.

These perivascular areas contain accumulations of phenotypically mature T cells (Pearse 2006).

Thymus development

Thymic organogenesis is a complex cascade of events involving reciprocal interactions between adjacent tissues, derived from all three embryonic germ layers (endoderm, ectoderm and neural crest-derived mesoderm). In mice, the thymus initially develops from the third and fourth branchial arches create a first visible thymic anlage as early as embryonic day 10-11 (E10-11). The pharyngeal region at this stage shows discrete swellings referred to as branchial arches (I – IV). The central lining of these arches forms four involutes pharyngeal pouches (pp, I – IV). Each of these pouches gives rise to specific tissues; the eustachian tube, tonsils, parathyroid-thymus, and ultimobrachial bodies, respectively. The common parathyroid–thymic primordium arises from a lateral outpocketing of endoderm of the third pharyngeal pouch under the influence of neural crest-derived mesoderm (Figure I). Although morphological and functional studies have demonstrated a broad heterogeneity among thymus epithelial cells (TECs), conflicting views as to the lineage relationship among these different TEC subpopulations have remained (Gill *et al.* 2003).

Ablation of cardiac neural crest cells that migrate into the pharyngeal arches results in malformations similar to those observed

in Velo Cardio Facial Syndrome (VCFS) / DiGeorge Syndrome (DGS) patients (Epstein 2001).

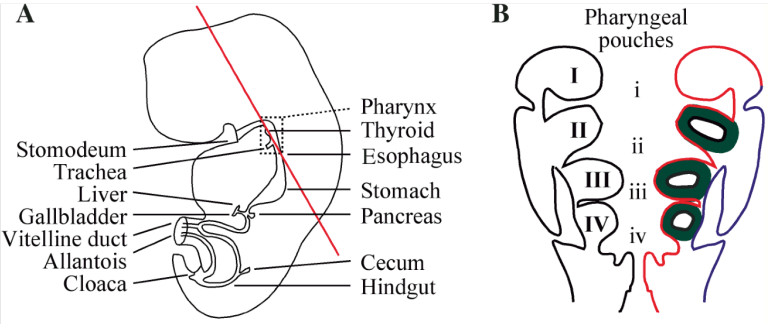


Figure I: Ontogenesys of thymus. (Gill *et al.* 2003)

Molecular control of thymic epithelial development

Analysis of spontaneous mutants and gene-targeted animals revealed that several transcription factors play an important role in early thymus organogenesis. Mutations in a number of genes, including Hoxa3, Eya1, Six1, Pax1, Pax3, Pax9, and TBX1 lead to thymus aplasia, or hypoplasia, or failure of the thymus lobes to migrate toward the chest during the embryonic development (Rodewald 2008).

A role for TBX1 in thymus organogenesis

TBX1 is a member of the phylogenetically conserved family of dosage sensitive transcription factors that share a common palindromic DNA-binding domain, named the T-box. TBX1 encodes

a nuclear transcription factor related to brachyury (a protein encoded by the T gene that is required for mesoderm formation). At later stages of development TBX1 can be detected in the thymus, and has been implicated in the complex DiGeorge syndrome phenotype (Lindsay *et al.* 2001).

Haploinsufficiency of other T-box family members, Brachyury or T, TBX3 and TBX5 are associated with congenital anomalies that occur with variable expressivity because T-box genes have been shown to act as homodimers and heterodimers to regulate transcription. This transcriptional synergy may be responsible for TBX1 haploinsufficiency in VCFS/DGS patients (Liao *et al.* 2004).

T CELL DEVELOPMENT

The development of mainstream T cells that bear the Ab form of the T-cell receptor (TCR) occurs exclusively within the thymus. Intrathymic T-cell maturation proceeds from the fetal liver or bone marrow-derived hemopoietic stem cells (HSCs) and occurs in a differentiation program readily characterized by changes in cell-surface phenotype, proliferation status, and functionality. Key events in T-cell development include lineage commitment, selection events, and thymic emigration.

T-cell lineage commitment

Blood-borne precursor cells that originate from Bone-Marrow (BM) stem cells populate the thymus. BM stem cells are not

committed to the T-cell lineage, because these cells have not recombined the variable (V), diversity (D) and joining (J) segment of the T-cell receptor (TCR) loci. T-cell commitment occurs after entry of the precursors into the thymus. As thymic immigrants enter the thymus in the CMJ, they differentiate in T-cell precursors and migrate outwards in the cortex subcapsular zone. The precursor thymocytes are often described as TN cells ($CD3^-CD4^-CD8^-$) and can be further subdivided based on their expression of CD44, CD25, CD117, and the IL-7aR chain CD127. In CMJ the most immature TN subset (TN1) requires many cytokines for survival and can be classified as $CD44^-CD25^-CD117^+CD127^-$. These cells acquire CD25 and CD127 to become $CD44^-CD25^+CD117^+CD127^+$ (TN2), where IL-7 is important for the growth and differentiation of T cells. IL-7 is a cytokine that is produced by stromal cells in lymphoid tissues and is required for development and homeostasis of most subsets of T cells (Offner and Plum 1998; Yarilin and Belyakov 2004). At this stage, TCRβ chain rearrangements are initiated in a process called β-selection. Decreased expression of CD117 and CD44 and the further decrease of CD25 have been used to determine the transition beyond the TN3 and TN4 stage respectively (Gill *et al.* 2003).

So in the subcapsular zone the TN4 $CD4^-CD8^-$ thymocytes proliferate, complete the β-selection and the TCRα rearrangements to differentiate into $CD4^+CD8^+$ (DP) cells that migrate back to the medulla. Surface expression of a mature CD3-TCRαβ complex is

first detectable on small non-proliferating DP thymocytes prior to positive selection.

TCR rearrangement and TCRBV repertoire

The potency of the immune system lies in its capacity to generate billions of different antigen receptors from multiple gene segments that are assembled by somatic recombination. Human peripheral T cell receptor (TCR) is membrane glycol-proteins, 95% of which are composed of α and β chain. α and β chains are created by the random joining of variable (V), diversity (D), and joining (J) gene segments by recombination, thus providing the diversity of the TCR recognition spectrum. (V)–(D)–(J) junctional-region complexity is increased by the addition or removal of nucleotides in joining sites, which further contributes to receptor-structure diversity. It has been reported that the TCR repertoire of variable gene segments in humans comprises more than 70 TCR αV (variable gene of TCRα chain) (Rowen *et al.* 1996) and about 57 TCR βV (variable gene of TCRβ chain) (Robinson 1991). These sequences were classified into 32 TCR αV families and 24 TCR βV families based on the nucleotide sequence similarity. Complementarity determining region 3 (CDR3) of TCRα chain is composed of the terminal of αV, the foreside of αJ and a nucleotide sequence inserted between them by terminal deoxynucleotidyl-transferase (TdT); β chain of TCR is derived from rearrangement of the genes "βV-βD-βJ-βC" fragments respectively.

The region containing highly variable junctions is the third of complementary determining regions (CDRs) that are seen crystallographically to contact antigen. CDR3 of TCRβ chain is composed of the terminal of βV gene fragment, βD gene fragment, the foreside of βJ gene fragment, and a nucleotide sequence inserted between V-D and D-J by TdT. Different T cell clones have different sequences or lengths of CDR3. Sequence of CDR3 region determines T cell receptor specificity, and one CDR3 sequence represents one T cell clone (Gorski *et al.* 1994; Hohn *et al.* 2002), moreover most of T cell clones binding to the same MHC peptide complex have the same CDR3 length. Since this region interacts most closely with the antigenic peptide, the diversity of the CDR3 amino acid sequences accounts for a wide array of antigen specificities within the functional T cell repertoire. The molecular interaction of interface peptides is similarly important in association between antigenic peptides and MHC molecules. This interaction limits heterogeneity of peptides that can bind to the products of a given MHC allele (Rammensee 1995). The length of the antigenic peptides is also restricted by interaction with MHC and with TCR (Rammensee 1995).

The immune response to infection involves the main expansion of small number of T cell clones, and consequently, a decrease in the TCR repertoire diversity. Accordingly to T cell clonal expansion, determination of the frequency of a specific CDR3 sequence may present the replication level of a specific T cell clone and, thus,

reflect the functional status of T cells. Spectratyping is a valuable method for the monitoring of antigen receptor diversity subsequent to lymphoid transplantation and to infection (Koga *et al.* 2003; Markert *et al.* 2003), providing information on antigen receptor diversity at the level of CDR3 length.

The size and diversity of the available human TCR repertoire are crucial in shaping immune responses to a given antigen. The theoretical diversity of the TCR repertoire assuming random V(D)J recombination is estimated to be 10^{15} (Davis and Bjorkman 1988), 1000-fold higher than the actual size of the human peripheral T cell compartment. TCRβ gene rearrangement or expression with the pre-Tα chains is necessary and sufficient for the progression of early alpha beta thymocyte differentiation from the $CD3^-CD4^-CD8^-$ TN3 to the $CD4^+CD8^+$ double positive stage.

Thymocyte selection

Following DP transition, developing thymocytes undergo extensive selection to ensure the mature T cells that are exported from the thymus are functional (self-MHC restricted) and self-tolerant. These processes are termed positive and negative selection and are dependent on lymphostromal interactions within the thymus. “Positive selection” process is a consequence of thymocytes which recognize self-peptide/MHC complex on cortical TEC (cTEC) at a low avidity, which results in rescue from cell death and the induction of differentiation leading to the generation of functionally competent

MHC class I-restricted cytotoxic $CD8^+$ T cells and MHC class II-restricted helper $CD4^+$ T cells; whereas thymocytes that recognize stromal cell-expressed peptide/MHC complex at a high avidity undergo “negative selection” process by the induction of apoptosis (Starr *et al.* 2003). Through these mechanisms, T-cell development in the thymus results in the generation of a self-tolerant T cell pool capable of recognizing foreign antigens in the context of self-MHC molecules.

So, T cells produced in the thymus are largely tolerant to the wide range of self-antigens expressed throughout the body, including peripherally expressed tissue-specific antigens (TSAs) such as antigens of the central nervous system, salivary gland, and pancreas. Many studies have now extended these initial observations and have shown that thymic epithelial cells express TSAs. The medullary thymic epithelial cells (mTECs) express these TSAs that are normally present only in specialized peripheral organs and are apparently not required for the direct function of mTECs. During negative selection, these encoded TSAs are presented by mTECs or dendritic cells to differentiating thymocytes as self antigens leading to the induction of tolerance by clonal deletion, functional inactivation or clonal deviation of self-reactive T cells (Peterson *et al.* 2008).

Aire is primarily, but not exclusively, expressed by medullary thymic epithelial cells (mTECs), and ensures developing thymocytes are exposed to a comprehensive view of ‘self’ permitting early

deletion of autoreactive cells before acquisition of effectors functions (Zuklys *et al.* 2000).

T regulatory cell lineage

An interesting cell population can be recognized inside the $CD4^+$ T-cell lineage, where analysis of expression of CD25 and the transcription factor FoxP3 allows the separation of $CD4^+$ T cells into conventional $CD4^+CD25^-FoxP3^-$ and regulatory $CD4^+CD25^+FoxP3^+$ (Treg) subsets. The earliest stage at which FoxP3 cells expression is detected is at the late $CD4^+CD8^+$ stage of thymocyte development and the majority are probably generated from cells that already underwent positive selection mainly along the $CD4^+$ single-positive lineage (Liston and Rudensky 2007).

Regulatory T cells (Treg) are indispensable for suppression of autoimmunity mediated by self-reactive T cells (Kim *et al.* 2007). Two origins have been described for Treg cells: the first is the thymus where $FoxP3^+$ cells are generate by positive selection of conventional $CD4^+$ T cells; the second is the periphery, where a number of triggers induce the expression of FoxP3 in converted Treg cells. Some of peripheral Treg cells arise in the thymus (natural Treg, nTreg), where up-regulation of the transcription factor FoxP3 is necessary for a subset of thymocytes to commit to the regulatory T cell lineage (Fontenot *et al.* 2003; Gavin *et al.* 2007). The transcription factor FoxP3 (forkhead box P3), a member of the

forkhead/winged-helix family of transcription factors, is a master regulator of Treg development and function.

Treg cells originate from thymocytes expressing T cell antigen receptors (TCRs) with an increased affinity for self-peptide–MHC complexes (Jordan *et al.* 2001). Importantly, mTEC, but not cTEC or thymic DC, are capable of expressing a broad range of tissue-restricted antigens via a poorly defined transcriptional mechanism dependent on nuclear factor Aire. This feature of mTEC led to the selection of FoxP3 Treg precursors on tissue-specific self-antigens displayed by mTEC preventing tissue-specific autoimmunity (Kyewski and Derbinski 2004). Strength of TCR signals during thymic selection, perhaps modified by costimulation or the nature of the antigen-presenting cell, may determine FoxP3 expression and, thereby, the regulatory phenotype of the T cell. In addition to stronger TCR engagement, signals sent through common γ-chain (γc)-containing cytokine receptors, IL-2R and the CD28 costimulatory receptor facilitate FoxP3-dependent Treg cell differentiation (Liston *et al.* 2007). FoxP3 functions by regulating a broad set of genes required for Treg suppressor activity and for proliferative and metabolic fitness (Zheng *et al.* 2007) and maintaining the lineage identity of peripheral mature Treg cells (Williams and Rudensky 2007). Studies using *ex vivo* isolated Treg suggest that FoxP3 protein amplifies or consolidates (makes more stable) features that were probably imparted by chronic TCR stimulation.

Numerous studies have suggested that under physiological conditions, the FoxP3-mediated Treg cell differentiation program is stable and hardly reversible, through repression of alternative differentiation pathways. In support of this notion, FoxP3 is able to override the TH17-promoting activities of the transcription factor RORγt (Ivanov *et al.* 2006), which at least at a message level is present in high amounts in Treg cells.

THE IMMUNE SYSTEM

We are continually exposed to organisms that are inhaled, swallowed, or inhabit our skin and mucous membranes. Penetrating of these organisms cause disease as result of both the pathogenicity of the organism and the integrity of host defence mechanisms. The immune system is organized as an interactive network of lymphoid organs, cells, humoral factors, and cytokines.

Immunity could be described in two parts, innate and the adaptive responses determined by the different speed and specificity of the reaction, although in practice interact. The term innate immunity is sometimes used to include physical, chemical, and microbiological barriers, but more usually encompasses the elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins) which provide immediate host defence. Adaptive immunity is the feature of the immune system of higher animals. This response consists of antigen-specific reactions through T lymphocytes and B-lymphocytes. Regarding the difference

between innate and adaptive immunity, the innate response is rapid but lack of specificity, while the adaptive response is precise, but takes several days or weeks to develop. The adaptive response has memory, so that subsequent exposure leads to a more vigorous and rapid response, but this is not immediate. The ability to develop and sustain populations of memory T cells after infection or immunization is a hallmark of the adaptive immune response and a basis for protective vaccination against infectious disease.

Adaptive Immunity

The characteristic of adaptive immunity is the use of antigen-specific receptors on T and B cells to drive targeted effector responses in two stages. First, the antigen is presented to and recognised by the antigen specific T or B cell leading to cell priming, activation, and differentiation, which usually occurs within the specialised environment of lymphoid tissue. Second, the effector response takes place, either due to the activated T cells leaving the lymphoid tissue and homing to the disease site, or due to the release of antibody from activated B cells (plasma cells) into blood and tissue fluids, and thence to the infective focus.

Antigen presentation and the MHC molecules

There are two ways in which antigen loading onto MHC can occur. The antigen may have been produced endogenously with the cell (such viral or tumour proteins) and is complexed with MHC class I through intracellular processing pathways. Because all nucleated

cells express MHC class I, this means that any such cell that is infected with a virus or other intracellular pathogen, or is producing abnormal tumour antigens can present these antigens with class I and be removed by cytotoxic attack. Alternatively, specialised professional antigen-presenting cells (APC) have taken up exogenous antigen by endocytosis and complexed with MHC class II molecules. APC include dendritic cells (the interdigitating dendritic cells of lymph nodes, veiled cells in the blood, and Langerhan's cells in the skin), B cells, and macrophages.

CD4 and CD8 have exclusive mechanisms of antigen recognition by TCR. CD4 lymphocytes recognise antigen presented with MHC class II, and endogenous antigens complexed with MHC class I molecules activate $CD8^+$ cytotoxic T cells. (Parkin and Cohen 2001)

Effector T cells

Upon activation by APCs and cytokines and costimulatory signals, $CD4^+$ or $CD8^+$ T cells differentiate into functionally distinct effector subsets.

Two major types of effector T cells are know, T helper (Th) and T cytotoxic (CTL), bearing either CD4 or CD8 molecules on their surface, respectively. $CD4^+$ Th cells are the cells of the immune response, recognising foreign antigen, and activating other parts of the cell-mediated immune response to eradicate the pathogen. They also play a major part in activation of B cells. $CD8^+$ cytotoxic cells

are involved in intra-cellular infection and possible antitumor activity.

T helper CD4$^+$ cells

Th cells play critical roles in the adaptive immune responses. They exert such functions mainly through secreting cytokines and chemokines that activate and/or recruit target cells. CD4 T lymphocytes can be subdivided into groups based on the cytokine profiles they produce. Naïve CD4 T cells differentiate into several functional lineages including T-helper 1 (Th1), Th2, Th0 and Th17 cells and regulator T cells (Treg) (see below).

Upon antigen encounter in the presence of IL-12 and IFN-γ, naïve CD4 cells can differentiate into T helper 1 (Th1) cells that are characterized by the production of IFN-γ, IL-2 and TNF-α (Abbas *et al.* 1996). Th1 cells mediate the Type-1 pathway ("cellular immunity") immune responses against intracellular pathogens. In humans, they play a particularly important role in resistance to mycobacterial infections. Dysregulated Th1 response to self-antigen often leads to tissue inflammation and organ-specific autoimmunity (Kuchroo *et al.* 2002).

Alternatively, antigen signalling in the presence of IL-4 induces the naïve CD4 cell population to develop into Th2 effectors cells. Th2 cells mediate the type-2 pathway ("humoral immunity") host defence against extracellular parasites including helminths, these cells are important in the induction and persistence of asthma and other

allergic diseases. The signature cytokines of Th2 cells are IL4, IL5, IL-9, IL-10, IL-13 and IL-25 (Abbas *et al.* 1996; Mosmann and Sad 1996).

Viral infections are known to predominantly induce Th1 or Type-1 immunity that promotes the activation of CD8 T cells and macrophage functions and drives B cell differentiation.

Interestingly, the cytokines of a particular Th subtype are able to further promote the expansion of that subtype population while simultaneously inhibiting the development of the other subset. This allows each Th subset to produce characteristic cytokines that in turn invoke the development of a distinctive effector response.

Furthermore a third type of Th lymphocyte that produce both Th1 and Th2 type cytokines (termed Th0) has been described in human and mouse systems (Mosmann *et al.* 1996).

T helper (Th) 17 cells represent a novel subset of CD4 T cells that are protective against extracellular microbes. Although the majority of the data concerning commitment to T cell lineages has been derived from mice, the presence of discrete IL-17 producing cells in humans has been detected; however, IL-17 is likely to be an important cytokine in the mediation of many inflammatory diseases and allograft rejection in humans (Vanaudenaerde *et al.* 2006). IL-17-producing CD4 T cells (Th17 cells) form a lineage separate from Th1 and Th2 cells, in fact, the production of Th17 cells is inhibited by factors that support Th1/Th2 differentiation (Annunziato *et al.* 2007). Th17 cells mediate immune responses against extracellular

bacteria and fungi. IL-17 can induce other proinflammatory cytokines (IL6, G-CSF, GM-CFS) and chemokines (IL-8, GROa), which are important in neutrophils recruitment, activation and differentiation.

T cytotoxic CD8$^+$ cells

CD8 T cells contribute to resistance against intracellular infections with certain viral, protozoan, and bacterial pathogens. Although they are known primarily for their capacity to kill infected cells, CD8 T cells elaborate a variety of effector mechanisms with the potential to defend against infection.

CD8 T cells bearing TCR specific for pathogen-derived antigens are selected to undergo clonal expansion. The specificity of this selection is driven by the interaction of the TCR peptides presented by MHC class I molecules on the surface of professional antigen-presenting cells (APC) (Germain 1994). Priming of CD8 T cells occurs in response to antigens that gain access to the cytosol of APC for processing by the endogenous MHC class I presentation pathway (Pamer and Cresswell 1998). Thus, CD8 T cells are activated in response to cytosolic infections with viruses, intracytoplasmic bacteria, and protozoa.

Activated CD8 T cells are able to induce cytolysis of infected cells by distinct molecular pathways: the granule exocytosis pathway, dependent on the pore-forming molecule perforin, or by the upregulation of FasL (CD95L), which can initiate programmed cell

death by aggregation of Fas (CD95) on target cells. Efficient lysis by the granule exocytosis pathway requires the coordinated delivery of perforin and granule enzymes, such as granzymes A and B, into the target cell (Heusel *et al.* 1994; Ebnet *et al.* 1995). These activate caspase enzymes that induce DNA fragmentation and cell apoptosis.

After stimulation through antigen receptor CD8 T cells also elaborate cytokines, including IFN-γ and TNF, as well as chemokines that function to recruit and/or activate the microbicidal activities of effector cells such as macrophages and neutrophils (Harty and Bevan 1999). Cytokines may also directly interfere with pathogen attachment or pathogen gene expression, or they may restrict intracellular replication. As with cytolytic effector mechanisms, expression of cytokine molecules by CD8 T cells is tightly regulated through TCR-dependent signals.

CD4 T-regulatory cells

Among $CD4^+$ T cell, Treg cells are specialized subsets able to control destructive immune response to pathogens and to prevent immune response against inappropriate targets, such as self-antigens or non-harmful external antigens. Suggested functions for Treg cells include: prevention of autoimmune diseases by maintaining self-tolerance, suppression of allergy, asthma and pathogen-induced immunopathology, feto-maternal tolerance, and oral tolerance. Two mainly classes of Treg are described according of their origin: naturally occurring Treg (nTreg) that originate in the thymus and

adaptive/induced Treg (iTreg) that are induced in the periphery upon different antigens and tolerogenic conditions which comprised of IL10-producing iTreg and TGF-β-producing iTreg.

The Forkhead box P3 (FoxP3) transcription factor has a critical role in the development of naturally occurring $CD4^+CD25^+$ T regulatory cells (nTreg), which are required for maintenance of immune tolerance. The scurfy mutation in mice, which is associated with a null mutation of the FoxP3 gene, results in complete loss of nTreg, autoimmunity, and premature death (Khattri *et al.* 2003). $CD4^+CD25^+FoxP3^+$ regulatory T cells were originally discovered in mice, but a population with identical phenotype has also been identified in humans (Sakaguchi *et al.* 2008). Humans lacking a functional FoxP3 gene also suffer from a systemic autoimmune disease know ad immune dysregulation, polyendocrynopathy, enteropathy X-linked syndrome (IPEX), where this disorder is associated with the impairment of nTreg function (Bacchetta *et al.* 2006).

Treg cells not only constitutively express CD25 and FoxP3 but also preferentially express other surface markers including CD103, glucocorticoid-induced tumour necrosis factor receptor (TNFR; GITR), cytotoxic T lymphocyte antigen-4 (CTLA-4), neuropilin-1, and lymphocyte activation gene-3 (Toda and Piccirillo 2006). However, these markers are only useful for distinguishing Treg cells from naïve conventional CD4 T cells because each can be induced by activation of conventional $CD4^+$ T cells. nTreg cells, especially in

human, stable express little or no IL-7Ra (CD127) even upon long lasting stimulation (Aerts *et al.* 2008). The absence of CD127 in combination with high levels of FoxP3 and CD25 provides an approach to identifying nTreg cells and separating them from other cells (Liu *et al.* 2006). Identification of nTreg cells remains problematic, because accumulating evidence suggest that all the discussed nTreg markers represent general T-cell activation markers rather being truly Treg-specific and new marker are proposed to identify those cell such as CD39, CD49d and more recently GARP (Borsellino *et al.* 2007; Battaglia and Roncarolo 2009; Kleinewietfeld *et al.* 2009). nTreg cells suppress mainly via cell-cell contact-dependent mechanisms, however activated Tregs, which express the high-affinity IL-2R at high levels, may absorb IL-2 from the surrounding and hamper the activation of other T cells (Sakaguchi *et al.* 2009). nTreg cells do not produce cytokine and are anergic upon TCR triggering (Toda *et al.* 2006), anergy is another unique feature of nTreg.

Although all activated T cells express CD25 and FoxP3, nTreg cells express the highest levels of CD25 and FoxP3 do so constitutively, whereas expression by conventional CD4 T cells can be transiently up-regulated and become effector T cells or stably expressed to become induced Treg (iTreg) (Roncarolo and Gregori 2008). Under TCR engagement by small amounts of antigen and cytokine stimulation, conventional $CD25^{-}CD4^{+}$ helper T cells can turn on FoxP3 and differentiate into an induced regulatory population

(iTreg). The iTreg (also termed Tr1 and Th3) produce cytokine such as IL-10 and TGF-β and show proliferative response upon in vitro stimulation (Bacchetta *et al.* 2002).

Tr1 cells can be generated in vitro and in vivo upon priming of naïve T cells with antigen in the presence of IL-10. Upon stimulation Tr1 cells produce high amounts of IL-10, considerable levels of IFN-γ, TGF-β and IL-5 but no IL-4 and very low levels or no IL-2 upon TCR-mediated activation (Roncarolo *et al.* 2001) (Table I); moreover FoxP3 can be upregulated to levels similar to those observed in activated $CD4^+CD25^+$ T cells although Tr1 cells suppress T cell response independently from FoxP3 expression (Roncarolo *et al.* 2006; Roncarolo *et al.* 2008). Tr1 are anergic upon antigenic polyclonal or specific stimulation (Bacchetta *et al.* 1994) but show low proliferative response in presence of IL-2 and IL-15 (Bacchetta *et al.* 2002).

Th3 cells are transforming growth factor (TGF-β) producing cells induced by oral tolerance (Chen *et al.* 1994), in these cells repetitive Ag stimulation results in sustained expression of FoxP3 and acquisition of regulatory function independent of surface expression of CD25 (Carrier *et al.* 2007).

Cytokine	$CD4^+$ T-cell subsets			
	Th0	Th1	Th2	Tr1
IL-2	+++	+++	+/−	+/−
IFN-γ	++	+++	+/−	++
IL-4	++	+/−	+++	−
IL-5	++	+/−	+++	++
IL-10	+	+	++	+++
TGF-β	++	++	++	+++

Table I: The typical cytokine production profile of human $CD4^+$ T- cell subsets. (Roncarolo *et al.* 2001)

B-cell

The major function of B-lymphocytes is the production of antibodies (Abs), glycoproteins belonging to gammaglobulin class, in response to foreign pathogens. This antibody production and binding specifically to a foreign substance or antigen, often is critical as a means of signalling other cells to engulf, kill or remove that pathogens from the body. Moreover B-cell may also act as antigen presenting cells interacting with T-cells.

B development in the bone marrow to mature antibody-secreting plasma cells in peripheral lymphoid tissues is a complex and tightly regulated process.

The earliest stages of normal B cell development are antigen-independent and occur in the bone marrow in response to stromal cell contact and cytokines. From the pre-B cell stage onwards, B cell development is dependent upon the successful assembly, and functional signalling through, pre-B and B cell receptor complexes. Central to this process is expression of the individual components of

the receptor and also the molecules that transduce signals from the receptor to initiate cellular events.

B lymphocyte development, in the bone marrow, starts with the somatic rearrangement of the heavy chain (HC) immunoglobulin (Ig) alleles. By pairing together diversity (DH), joining (JH), and variable (VH) region DNA segments, many pro-B cells, each with a single but unique HC allele, are produced. Those cells undergo clonal expansion and proceed to the pre-B stage, before repeating the whole rearrangement process for the light chain (LC) Ig alleles. A productive LC rearrangement results in surface expression of IgM, which acts as the B cell receptor (BCR) for antigen for the immature B cell. During development, any B cells bearing strongly self-reactive Ig receptors are removed (this process is called tolerization) either by clonal deletion, by functional inactivation, or by receptor editing. In this last process, new LC rearrangements revise the antigen specificity of the receptor (Tze *et al.* 2005).

After antigen-independent development in the bone marrow, immature B cells leave the bone marrow and are transported by the bloodstream to secondary lymphoid organs.

In secondary lymphoid organs immature B cells IgM^+, also referred as "transitional" (T1 and T2), acquire cell surface IgD as well as CD21 and CD22 for the progression via transitional into mature B cells (Loder *et al.* 1999). Here mature naïve IgD^+IgM^+ B-cell pool when stimulated by antigen in a germinal center (GC) reaction in a

T-cell dependent manner develop into long-lived plasma cells or memory B cells.

CD40/CD40L interaction is crucial to the B-cell development T-dependent during a GC reaction. This signal also has a role in the development of germinal centres (GC) and the survival of memory B cells. The CD40L expressed by activated T-cells provides signals to B cells that induce proliferation, somatic hypermutation (SHM) of V_H genes, class switch recombination (CSR) and produce higher affinity IgD^-IgM^- class-switched B-cell clones that form the memory compartments of humoral immunity (Wykes 2003; LeBien and Tedder 2008).

CD27 expression identifies all human memory B cells. The human memory B cell population is heterogeneous, comprising cells that are either $CD27^+IgM^+IgD^+$ (~40-50%) or $CD27^+IgM^-IgD^-$ (~30-40%; corresponding to the classically defined isotype-switched IgG^+ or IgA^+ memory cells) (Tangye and Good 2007).

PRIMARY IMMUNODEFICIENCY DISEASE

Primary immunodeficiency disease (PIDs) consists of a group of more than 100 inherited conditions, mostly monogenic. Clinical presentation is highly variable, including increased susceptibility to infections, allergy and autoimmunity manifestations. In many cases, inherited disease have led to identification of new genes that are crucial in immune cell development or effectors function or that contribute to prevent overt autoimmune disease. In those manner the

related genes alteration has generated a profusion of information about the immune system and many aspects of the development, function and regulation of both innate and adaptive immunity (Fischer 2004). The international Union of Immunology Societies (IUIS) has produced regular reports on the classification of primary immunodeficiency disease (PIDs), with most recent update in 2007 (Geha *et al.* 2007).

DiGeorge Syndrome

DiGeorge Syndrome (DGS) is one of the most common chromosomal disorders, with an estimated prevalence of 1 in 4000 to 6000 (Botto *et al.* 2003). The syndrome arises from a defect in the differentiation of the third and fourth pharyngeal pouches during embryologic development. The 22q11.2 deletion syndrome results most commonly from heterozygous chromosome deletions of 3Mb in the chromosome 22q11.2 region (about 90% - 95% cases) (Sullivan 2004) that cover approximately 25-30 genes; less common is a 1.5 Mb deletion (about 7%) (Carlson *et al.* 1997; Scambler 2000; Lindsay 2001; Yamagishi 2002). The clinical presentation of DGS is characterized by both the uneven penetrance of distinct disease components and a variable expressivity of the pathology.

22q11.2 deletions can be reliably detected with fluorescent in situ hybridization (FISH) probes (Yakut *et al.* 2006; Oh *et al.* 2007). Stochastic, environmental and genetic modifiers likely account for the wide variation seen in 22q11.2 deletion syndrome, but no

functional explanation currently exists (Bittel *et al.* 2009). The availability of a mouse model for DGS has been extremely helpful, not only to identify the precise molecular cause of the syndrome but also to characterize molecules that may act as genetic modifiers.

TBX1 encodes a T-box transcription factor, which is known to have an essential role in early vertebrate development. TBX1 has been suggested as a candidate gene for 22q11.2 deletion syndrome (Gong *et al.* 2001; Jerome and Papaioannou 2001; Lindsay *et al.* 2001). Indeed mice with deletions homologous to the human 22q11.2 display most of the cardiac and pharyngeal arch defects observed in the 22q11.2 deletion syndromes. The clinical phenotype in children is variable and severity of symptoms range from mild, near normal to life threatening. Hemizygous deletions of the chromosome 22q11.2 region typically result in conotruncal cardiac anomalies, alterations of thymus (hypoplastic or absent) and parathyroid glands, moderate to severe immune deficiency, developmental delay and behavioural problems, facial dysmorphia, palatal dysfunction and feeding difficulties (Kobrynski and Sullivan 2007; Ben-Shachar *et al.* 2008).

35-90% of patients clinically diagnosed with DiGeorge Syndrome (cardiac anomalies, hypoparathyroidism, immunodeficiency) and 80-100% with velocardial syndrome (pharyngeal dysfunction, cardiac anomaly, dysmorphic facies) have the hemizygous deletion (Kobrynski *et al.* 2007). Table II review clinical features of children with the 22q11.2 deletion syndrome.

	Frequency of finding
Cardiac anomalies	49–83%
Tetralogy of Fallot	17–22%
Interrupted aortic arch	14–15%
Ventriculoseptal defect	13–14%
Truncus arteriosus	7–9%
Hypocalcaemia	17–60%
Growth hormone deficiency	4%
Palatal anomalies	69–100%
Cleft palate	9–11%
Submucous cleft palate	5–16%
Velopharyngeal insufficiency	27–92%
Bifid uvula	5%
Renal anomalies	36–37%
Absent or dysplastic	17%
Obstruction	10%
Reflux	4%
Ophthalmological abnormalities	7–70%
Tortuous retinal vessels	58%
Posterior embryotoxon (anterior segment dysgenesis)	69%
Neurological	8%
Cerebral atrophy	1%
Cerebellar hypoplasia	0·4%
Dental	
Delayed eruption, enamel hypoplasia	2·5%
Skeletal abnormalities	17–19%
Cervical spine anomalies	40–50%
Vertebral anomalies	19%
Lower limb anomalies	15%
Speech delay	79–84%
Developmental delay in infancy	75%
Developmental delay in childhood	45%
Behaviour or psychiatric problems	9–50%
Attention deficit hyperactivity disorder	25%
Schizophrenia	6–30%

Table II: Clinical findings in patients with chromosome 22q11.2 deletion syndrome. (Kobrynski *et al.* 2007).

Immunological defects in DiGeorge syndrome.

Immunological defect affect about 75 – 80 % of patients with 22q11.2 deletion syndrome (Kobrynski *et al.* 2007) and involve primary T cell compartment. Immune deficiency in 22q11.2 deletion syndrome occurs because of thymic aplasia or hypoplasia and the consequent missing or impaired function of this crucial immune organ in lymphocyte development (Sullivan *et al.* 1998; Lindsay 2001). Similarly to the phenotypic features, a wide variability to the immunological defect exists in patients with DiGeorge syndrome, ranging from the absence of T cells to normal T cells levels. On the basis of the immunologic findings, DGS patients can be divided in "complete DiGeorge" (cDGS) and "partial DiGeorge" (pDGS). Patients with complete absence of thymus (<0.5 - 1% of patients) exhibit severe T cell deficiency with severe combined immunodeficiency phenotype. On the basis of immunological alterations the term "complete DiGeorge" (cDGS) is used to describe the population of infants with DiGeorge anomaly who are athymic, which have no or very few detectable T-cells in peripheral blood or no T-cell responsiveness to mitogens (Muller *et al.* 1988; Markert *et al.* 2003; Piliero *et al.* 2004). Complete DiGeorge is uncommon and usually presents with severe infections in the first months of life caused by opportunistic pathogens. Several strategies have been applied for immune reconstitution in DiGeorge patients, including thymus or haematopoietic stem cell transplantation HSC (Bowers *et al.* 1998; Markert *et al.* 1999; Bensoussan *et al.* 2002). Predictably,

more naïve T cells and recent thymic emigrants are present in patients treated with thymus transplant. The largest experience in treatment of complete DGS to date has been with thymus transplant, Markert et al. recently reported a series of 54 patients with complete DGS who underwent thymus transplant with generally good outcomes (Markert *et al.* 2004). However, the majority of patients with 22q11.2 syndrome belongs to the category characterized as "partial DiGeorge" (pDGS) (Muller *et al.* 1988; Jawad *et al.* 2001). These patients have thymic hypoplasia, exhibit mild to moderate deficits in T-cell count but fairly normal T cell proliferative response to mitogens and near-normal levels of naïve T cells. Frequent and severe perturbations of T cell receptor (TCRBV families) have been previously described in patients with partial 22q11.2 deletion syndrome mainly with to regard $CD8^+$ T cell subset (Piliero *et al.* 2004; Cancrini *et al.* 2005). In healthy subjects, occasional expansions are detected exclusively within the CD8 subset (Hingorani *et al.* 1993). These expansion gradually accumulate during life and may represent: the result of antigen-driven clonal expansion due to immune responses to viral infections (Argaet *et al.* 1994; Khan *et al.* 2002) or defect in the mechanism regulation T cell rearrangement and maturation (Brooks *et al.* 1999). Because the diversity of peripheral T cell repertoires is maintained by the continuous generation in the thymus of new naïve T cell, in partial 22q11.2 patients the altered TCR repertoire of $CD8^+$ T cell in

children with pDGS reflects a developmental defect rather than an antigenic stimulation (Cancrini *et al.* 2005).

The immunodeficiency in pDGS patients is unusual among T-cell disorders because it is a failure of the matrix in which the T cell develops, rather than an intrinsic defect of the T cell itself, that is responsible for the deficit (Markert *et al.* 1999). Furthermore, T-cell development in an abnormal thymus may also result in impaired central tolerance and escape of self-reactive T cells, which would normally be deleted. Indeed as showed in mice, neonatal thymectomy results in the development of autoimmune disease (Asano *et al.* 1996). On the other hand it could not be excluded that autoimmunity may be a consequence of both marked lymphopenia and increase of infections. Indeed autoimmune phenomena (autoimmune cytopenias, autoimmune arthritis and autoimmune endocrinopathy) have frequently been described in patients with DiGeorge syndrome (10–20 % of del22q11.2 patients) (Sullivan *et al.* 1997; Jawad *et al.* 2001; Gennery *et al.* 2002).

Previous studies showed an overall reduction of nTreg compartment in pDGS patients when compared with healthy donors, although the absolute count or proportion of these cells in the patients with autoimmune disease was not different compared with patients without autoimmune disease (Sullivan *et al.* 2002; McLean-Tooke *et al.* 2008).

Although immunodeficiency of pDGS is classically described as cell mediated, alterations in the humoral compartment, such as IgA

deficiency, hypogammaglobulinemia, selective antibodies deficiency and reduced level of Memory $CD27^+$ B cells have reported in these patients (Finocchi *et al.* 2006).

Chronic Granulomatous Disease

Chronic Granulomatous disease (CGD) is an inherited disorder caused by defects of the phagocyte nicotamide dinucleotide phosphate (NADPH) oxidase (also referred to as the respiratory burst oxidase). Phagocytosis is normally accompanied by marked increase in oxidative metabolism and studies have shown that NADPH oxidase was the respiratory enzyme responsible for bactericidal activity. Superoxide generated during the phagocyte respiratory burst is the precursor to numerous microbicidal oxidants, including hydrogen peroxide (H_2O_2) and myeloperoxidase-catalyzed formation of hypoclorous acid. Respiratory burst-derived oxidants are an important component of the innate immune response, and patients with CGD suffer from recurrent and life-threatening bacterial and fungal infections (such as *Staphylococcus aureus*, *Serratia marcescens* and *Aspergillus species*) and from abnormal exuberant inflammatory responses leading to granuloma formation in multiple organs (Babior 1999; Segal *et al.* 2000; Dinauer 2005). Due to these severe infections most of the patients are diagnosed in early childhood and overall the disorder has an estimated incidence between 1:200000 and 1:250000 live births (Winkelstein *et al.* 2000).

The NADPH oxidase is a phagosomal and plasma membrane-associated enzyme complex that is inactive in resting neutrophils and rapidly assembled when cells are activated by a variety of inflammatory stimuli (Nauseef 2004). The structure of NADPH oxidase is quite complex, consisting of two membrane-bound elements (gp91^{phox} and p22^{phox}), three cytosolic components (p67^{phox}, p47^{phox} and p40^{phox}), and a low molecular weight G protein (either RAC2 or RAC1) (Babior 1999).

Mutations can occur in one of four NADPH oxidase subunits, which give rise to X-linked or autosomal recessive CGD. Most patients with CGD (65% of cases) result from defect in the *Cybb* X-linked gene encoding the gp91^{phox} subunit of flavocytochrome b_{558}, located at Xp21.1. The remaining mutations are autosomal recessive: mutation in the *Ncf1* gene which encodes the p47^{phox} represent about 25% of CGD cases; mutation in the *Ncf2* and *Cyba* which encoding p67^{phox} and p22^{phox} are responsible for the 5% of CGD cases respectively (Assari 2006). Genetic defects in Rac or p40^{phox} have been reported in few CGD patients (Dinauer 2005) (Figure II).

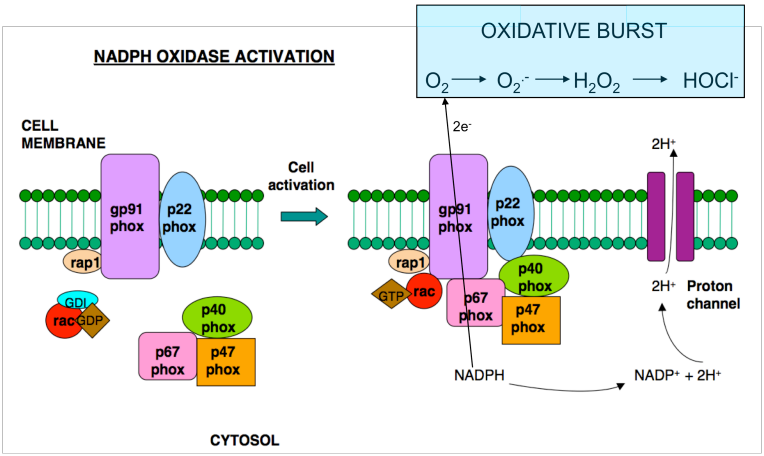


Figure II: NADPH oxidase subunits and their activation. Adapted from Tracy Assari (Assari 2006).

Currently, the treatment is basically preventive and is based on rigid infectious prophylaxis provided by lifelong co-administration of an antibacterial and an antifungal agent. However, although the progress recently made in detecting and treating infections, CGD remains characterized by a high prevalence of mortality. Allogenic haematopoietic stem cell transplantation has been successfully employed in CGD and is currently the only proven curative treatment (Seger 2008).

However, CGD patients not only suffer from recurrent infections, but also present with an increased frequency of inflammatory and autoimmune disorders (Cale *et al.* 2000; Brown *et al.* 2003). In the light of the various hyperinflammatory states observed in CGD, ROS production by NADPH is likely to play an active role in the resolution of inflammation. Possible mechanisms involved in CGD

hyperinflammation include: decreased degradation of phagocytosed, redox-dependent termination of proinflammatory mediators and/or signalling, redox-dependent cross talk between phagocytes and lymphocytes and decreased apoptosis of neutrophils. Decreased degradation of phagocytosed material could be due to deficient generation in CGD phagocytes, so phagocytosed material could accumulate in deficient phagocytes leading to persistent cell activation (Schappi *et al.* 2008a). Reactive oxygen species (ROS)-dependent attenuation of Ca^{2+} signalling, through regulation of membrane potential, may be impaired in CGD patients (Geiszt *et al.* 2001), contributing to enhanced inflammation. Reduced expression of ROS in CGD patients creates signalling alterations, which favour pro inflammatory responses. Indeed CGD phagocytes release inflammatory mediators such TNF-α and IL-8 after stimulation (Rada *et al.* 2003; Lekstrom-Himes *et al.* 2005) and have an impaired ability to produce anti-inflammatory mediators such as TGF-β and prostaglandin (Brown *et al.* 2003).

No relevant alterations regarding T cell compartment are know in literature. Only few investigations have analyzed T-cell population in Chronic Granulomatous Disease describing diminished T cells number (Heltzer *et al.* 2002) and memory T cell (Hasui *et al.* 1993). While PBMC proliferative response was show similar to controls, although the mean was lower in the patients compared to control (Heltzer *et al.* 2002; Salmen *et al.* 2007).

Noteworthy NADPH oxidase play a role not only in the granulocyte compartment, indeed in 2004 Williams' group found a phagocyte-type NAPDH oxidase expression in mature T-cell (Jackson *et al.* 2004). Indeed recently a ROS mediated T-cell apoptosis upon cytokine deprivation was described by Purushothaman (Purushothaman and Sarin 2009).

Furthermore Salmen *et al.* (Salmen *et al.* 2007) observed beside a normal number of T cells a diminished expression of CD40L in T cells from CGD patients following stimulation with PHA, suggesting a deficient T-cell activation response.

Hypogammaglobulinemia as result of Primary B Cell Immunodeficiency

Hypogammaglobulinemia generally can be divide into primary/genetic causes or secondary causes due to other many disorders, such malignancy, and various treatment (Table III).

Drug Induced
Antimalarial agents
Captopril
Carbamazepine
Glucocorticoids
Fenclofenac
Gold salts
Penicillamine
Phenytoin
Sulfasalazine
Genetic Disorders
Ataxia Telangiectasia
Autosomal forms of SCID
Hyper IgM Immunodeficiency
Transcobalamin II deficiency and hypogammaglobulinemia
X-linked agammaglobulinemia
X-linked lymphoproliferative disorder (EBV associated)
X-linked SCID
Some metabolic disorders
Chromosomal Anomalies
Chromosome 18q- Syndrome
Monosomy 22
Trisomy 8
Trisomy 21
Infectious Diseases
HIV
Congenital Rubella
Congenital infection with CMV
Congenital infection with Toxoplasma gondii
Epstein-Barr Virus
Malignancy
Chronic Lymphocytic Leukemia
Immunodeficiency with Thymoma
Non Hodgkin's lymphoma
B cell malignancy

Table III: Differential Diagnosis of Hypogammaglobulinemia. From **ESID**.

The term primary B cell immunodeficiencies encompass a heterogeneous group of disorders that share the marked reduction or absence of serum immunoglobulins. Antibody deficiency syndromes as a whole make up the greatest proportion of PID diagnoses-up 67% to 77% of all Primary Immunodeficiency Disease, as recently published by the European and Australian registries (Kirkpatrick and Riminton 2007; Gathmann *et al.* 2009).

In recent years it has become increasingly clear that interpreting the consequences of alterations in DNA in these inheritances but may be more complex than initially recognized. Although specific mutations in genes of interest may account for some of this variability (Lopez-Granados *et al.* 2005; Broides *et al.* 2006) other factors as genetics, the age of the patients, environmental exposure plays a role as well. Defective genes that are mainly responsible for antibody deficiencies may be intrinsic to the B cell lineage (Tsukada *et al.* 1993; Yel *et al.* 1996), or may encode signal transduction molecules expressed by T cells (Thusberg and Vihinen 2007; Yong *et al.* 2009).

There are three major categories of antibody deficiencies syndrome: *(a)* defects in early B cell development, *(b)* hyper-IgM syndromes (also called class switch recombination defects), and *(c)* Common Variable Immunodeficiency (CVID). Patients in both groups generally have marked reduction in serum IgG and IgA titers. All the antibody deficiencies are associated with an increase susceptibility to bacterial infection especially with encapsulated bacteria (*Streptococcus pneumonie*, *Haemophilus influenza and Neisseria)* (Bruton 1952; Oksenhendler *et al.* 2008). A summarized picture of Primary B cell immunodeficiencies is showed in Figure III.

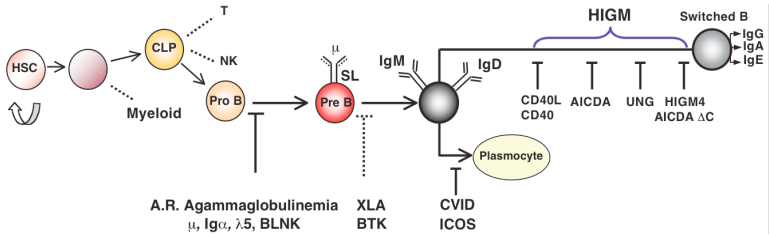


Figure III: B-cell development and related primary B-cell immunodeficiencies. (Fischer 2004)

Defect in early B cell development

Defect in early B cell development are characterized by onset of recurrent bacterial infections after their maternal immunoglobulins (Igs) have been lost, profound hypogammaglobulinemia, markedly reduced or absent B cell in the peripheral circulation, and (in the bone marrow) a severe block in B cell differentiation before the production of surface immunoglobulin-positive B cells. Approximately 85% of patients with defects in early B-cell development suffer from X-linked agammaglobulinemia (XLA), a disorder caused by mutations in the cytoplasmic Bruton's tyrosine kinase (Btk)(Conley *et al.* 2005). Approximately 15% of patients with congenital agammaglobulinemia and absent circulating B cells do not have a mutation in Btk but have defects in components of the pre B cell receptor including the downstream scaffold protein BLNK (Tan *et al.* 2001; Conley *et al.* 2009), m heavy chain (IGHM), the signal transduction molecules Igα (*CD79A*) (Minegishi *et al.* 1999) and Igβ (*CD79B)* (Ferrari *et al.* 2007); and λ5 (*IGLL1*) (Minegishi *et al.* 1998) (Figure IV). Clinically, these patients with the autosomal

recessive form of agammaglobulinemia (ARA) and related defects in these proteins are not easily distinguishable from patients with XLA. However, they tend to be younger at the time of diagnosis, and whereas most patients with XLA have a small number of B cells in the peripheral circulation, these cells are not found in patients with defects in m heavy chain or Igα (Conley *et al.* 2005). In approximately 5% to 10% of all patients with defects in early B-cell development, no clear molecular defect ha been identified.

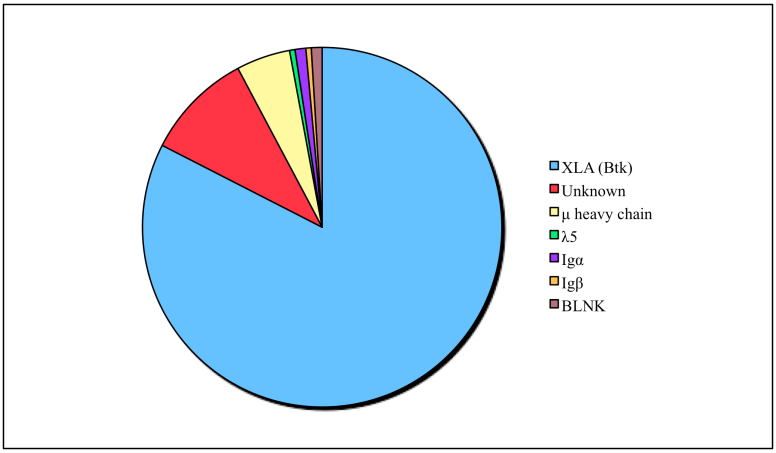


Figure IV: Percentage of patients with early defects in B cell development who have mutations in each of the genes shown. (Conley *et al.* 2009)

BTK Deficiency/X-linked Agammaglobulinemia

X-Linked agammaglobulinemia (XLA), or Bruton's agammaglobulinemia, is a X-linked genetic disorder first described in 1952 (Bruton 1952). XLA caused by mutations in the gene coding for the Bruton's Tyrosine kinase (Btk), which is implied in the development of B cells. Mutations of Btk gene cause the incomplete differentiation of B cell precursors to mature B cells or the inefficient expansion of pre-B cells into later B cell stages (Campana *et al.* 1990; Tsukada *et al.* 1993). There is significant variability in Btk mutations, with more than 600 different mutations in Btk (Valiaho *et al.* 2006) and no single mutation accounting for more than 3% of patients in one series. XLA diagnosis in all these patients was based on family history, typical clinical and immunological findings, including recurrent otitis media, sinusitis, bronchitis and pneumonia, an almost total lack of peripheral blood B cells (<2%), and very low levels of serum immunoglobulin isotypes (IgG < 2 g/l, IgA and IgM < 0,2 g/l). XLA diagnosis is confirmed in male patient with mutations of the BTK gene and less than 2% $CD19^+$ B cells (http://www.esid.org). Patients usually become symptomatic in infancy or early childhood after their maternal immunoglobulins (Igs) have been lost.

Common Variable Immunodeficiency

CVID is an heterogeneous group of predominantly antibody deficiency disorders that make up the greatest proportion of patients

with symptomatic primary hypogammaglobulinemia, with an estimated population prevalence of between 1 in 1000 and 1 in 50000 (Societies 1999). All clinical immunologists would agree that the term CVID includes a heterogeneous group of disorder characterized by recurrent infections and failure to make antibody to vaccine antigens (Cunningham-Rundles and Bodian 1999; Quinti *et al.* 2007; Yong *et al.* 2008). Clinically it is definited by the presence of recurrent infection, a reduction in IgG (of al least two standard deviations below the mean) and at least one other Ig isotype, a failure to generate a significant specific antibody response after vaccination or natural infection after, onset of immunodeficiency at greater than 2 years of age after other known genetic or acquired causes of hypogammaglobulinemia have been excluded (http://www.esid.org). Over the years there have been several attempts to subdivide patients with CVID into groups based on clinical or laboratory findings (Chapel *et al.* 2008; Wehr *et al.* 2008) but in general, these classifications have not been helpful in determining the genetic aetiology of CVID.

The past few years have been seen the discovery of mutations/polymorphisms in five genes that result/contribute to a CVID phenotype. Grimbacher *et al.* have described nine patients with the same homozygous deletion in the gene encoding inducible costimulator ([ICOS] gene: *ICOS*) on T cells (Grimbacher *et al.* 2003; Salzer *et al.* 2004). Mutations in the gene encoding CD19 also cause an immunodeficiency that could be mistaken for CVID. Van

Zelm *et al.* described four patients from two unrelated families, a Turkish family and a Colombian family, who had normal numbers of B cells when CD20 was used as the B cell specific marker, but had no CD19 B cells (van Zelm *et al.* 2006). Heterozygous mutations in the TNF receptor family member TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor) can be found in up to 10% of patients with CVID (Salzer *et al.* 2005; Pan-Hammarstrom *et al.* 2007). BAFF-R deficiency has so far been identified in only one patient 60-year-old man (Warnatz *et al.* 2004).

LABORATORY TECHNIQUES IN IMMUNOLOGY

In these thesis three techniques has been used to study the development of the adaptive immune system. These techniques are both methods and specialized experimental protocols use to for inducing, measuring, and characterizing immune system.

Flow Cytometric Analysis

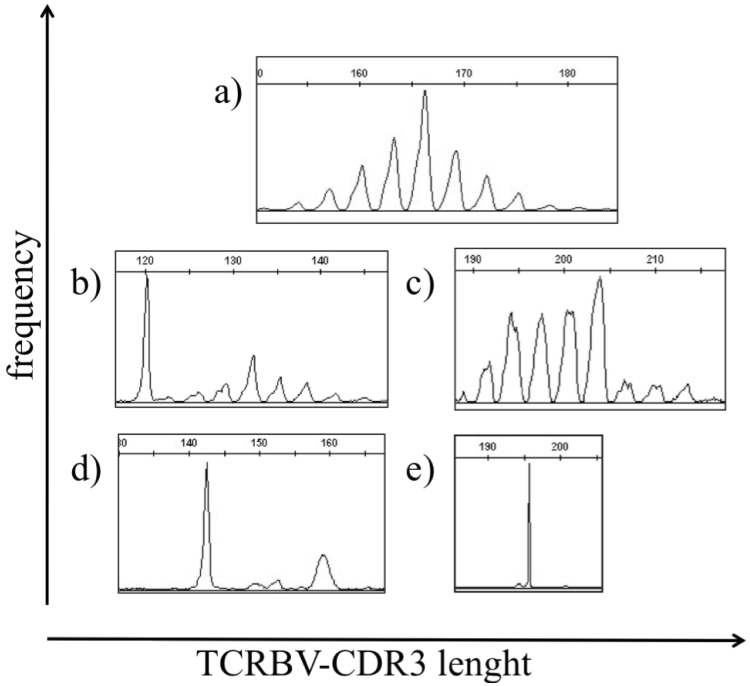
Flow cytometry is an established technology in the clinical laboratory. One example of its importance is the immunofluorescence-based flow cytometry for the identification and enumeration of lymphocyte subpopulations. Flow cytometry provides rapid analysis of multiple characteristics of single cells. The information obtained can be both qualitative and quantitative.

CDR3 Spectratyping

Spectratyping is a valuable method for the monitoring of TCR repertoire subsequent infection and lymphoid transplantation. Severe immune disruptions, either caused by genetic lesions disrupting normal developmental processes or acquired during life as a result of infections or leukaemia, result in loss of antigen receptor diversity. In particular the immune response to infection involves the manifold expansion of a small number of T cell clones, and consequently, a

decrease in the TCR repertoire diversity. Spectratyping is a RT-PCR method, which provides information on antigen receptor diversity at the level of CDR3 length. The point is not that length diversity itself is of particular relevance (though it might be), but that length heterogeneity and it's mathematical distribution of frequencies is representative of overall sequence heterogeneity, and consequently the ability of T cells to recognize a vast array of antigens (Kepler *et al.* 2005).

The classical ways to represent spectra data of CDR3s lengths are histogram, where pulled CDR3s lengths for each Vβ family are related with their frequency.



CDR3s' spectra: a) normal peak distribution in healthy donors (p); b) and c) polyclonal/altered peak distribution (pa); d) and e) oligo or monoclonal distribution (SK) indicate T cell expansion.
Healthy donors TCR spectra exhibit polyclonal Gaussian patterns of CDR3 lengths for every single rearranged Vβ gene, while during both pathogens' infections or genetic defect of rearrangement a reduction in TCR repertoire diversification is observed.

Lymphoproliferation assay

In vitro culture of peripheral blood mononuclear cells (PBMC) with mitogens either plant lectins (PHA, PWM) or monoclonal antibodies (OKT3), antigens is one of the oldest and most widely applied methods for assessing lymphocyte response to stimulation and therefore impaired T-cell function.

After incubation in vitro with mitogens or specific antigens for 3 or 7 days respectively lymphocytes proliferate. Lymphoproliferation is then measured by ^{3}H-thymidine incorporation. The rate of proliferation, expressed as the CPM of radioactivity incorporated into the DNA of dividing cells, provides an indication for general lymphocyte activity or responsiveness stimula. A "stimulation index" (SI) has been defined as the ratio between stimulated CPM and unstimulated PBMC:

$$SI = \frac{cpm\,of\ \ PBMC\ with\,mitogen}{cpm\,of\ \ spontaneous\,PBMC\ \ proliferation}$$

Reduced thymidine uptake may be due to: a) reduced rate of division of all T cells, b) normal responsiveness of only a fraction of T cells, and c) a smaller representation of T cells among PBMC, or a combination of these (Stone *et al.* 2009).

AIMS OF THE STUDY

The main aims of this study can be summarized as:

I. To clarify the pathogenesis of the immunological defect in pDGS and CGD patients.

II. To identify possible biological markers predictive of susceptibility to infections and/or autoimmunity, correlating immunologic in vitro studies with patients' clinical features.

In order to achieve these aims we will evaluate:

1. Dynamic of immune reconstitution in pDGS patients with the age and T cell compartment in CGD patients, through analysis of:
    a. Phenotype and immunologic function through standardized techniques.
    b. Distribution of TCR repertoire of $CD4^+$ and $CD8^+$ T-cell subsets through the molecular analysis CDR3 heterogeneity by spectratyping.
2. B cell memory subsets by immunophenotyping of peripheral blood cells.
3. Frequency of natural regulatory T-cell (nTreg).

MATERIALS AND METHODS

Study population

Thirty patients with chromosome 22q11.2 deletions (DGS), twelve patients with Chronic Granulomatous Disease (CGD), twenty-four patients with Hypogammaglobulinaemia most of them definite as CVID (3 patients with X-linked hypogammaglobulinaemia XLA) (according to the ESID diagnostic criteria) and twenty newborn CMV congenital infected patients were studied. These patients were compared with age-matched healthy controls as described in the following sections. Parental permission was obtained for all tested subjects according to the procedures established by the Ethical Committee of our Institution.

Lymphoproliferation assay

Methods:

A number of agents as mitogens are used to induce lymphocytes proliferation. Mitogens as phytoemagglutinin (PHA, PWM) activates cells in unspecific way and OKT3 induce T lymphoproliferation via simulated immune reactions (monoclonal antibodies specific for the CD3 component of the T-cell antigen receptor). In this procedure, lymphocytes are isolated by Ficoll-Paque PLUS density gradient centrifugation and cultured (200000 cells/well) in triplicate with or without stimuli for 3 days (mitogens) or 7 days (antigens) in cell cultures RPMI-1640 enriched with 10% human serum. Cell cultures

were pulsed with 2.5μCi/ml methyl tritiated thymidine ([^{3}H]) for the last 6 hours and harvested onto glass fibre. Then the glass filter was soaked in scintillation fluid and counted for tritium in a b-Counter scintillator (Canberra Packard Instrument Company, Meriden, CT, and U.S.A.). The results are expressed as a stimulation index (SI).

Materials:

- Ficoll-Paque PLUS (GE Healthcare, Sweden)
- RPMI-1640 with L-glu culture medium (Sigma, St. Louis, MO) supplemented with 10% AB Human serum (Sigma), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Sigma)
- 96-well, flat bottom (Becton Dickinson, Falcon, NJ, USA)
- Glass fibre filters (Packard, MI, Italy)
- [^{3}H] (GE Healthcare, Amersham)
- PHA 5 μg/ml (Sigma, MI, Italy)
- PWM 0.35 μg/ml (Sigma, MI, Italy)
- OKT3 3 μl (stock supernatant obtained from OKT3 cell line, ATCC-Sigma, MI, Italy)

CDR3 Spectratyping

Methods:

CDR3 length spectratyping was employed to examine peripheral $CD4^+$ and $CD8^+$ T-cell populations for their CDR3 length repertoires.

24 individual Vb-family specific amplifications are performed using RT-PCR. The size distributions of TCR-CDR3 fragments in each Vb family are then resolved by capillary electrophoresis.

Fresh PBMC isolated with Ficol-Hypaque density gradient centrifugation, were fractionated into $CD4^+$ and $CD8^+$ subsets by incubating with the specified anti-CD4 or anti-CD8 monoclonal antibody-coupled magnetic beads for 1 hour at 4°C on a rotating shaker, according to standard protocol. Total RNA was extracted by TRIzol® according to mildly modified manufacturing instructions.

cDNA was synthesized and amplified with a SuperScript One-Step RT-PCR kit (Invitrogen, Milan, Italy) as previously described by 24 different primers for unique 5'VB sequences in combination with a primer for the TCRBV region 3' Cb primer FAM-labeled. Final mix was heated at 50 °C for 20 min followed by 94 °C for 5 min and cDNAs were amplified by 32 cycles: 95 °C 45'', 55 °C 45'', 72 °C 45'' and finally extended at 72 °C for 10 min in a GeneAmp 9600 DNA thermal cycler (Perkin Elmer Cetus).

PCR products undiluted or diluted were added to a mix volume of Formamide and ROX Gene Scan 500 Size Standard dye and then plated on a 96 well MicroAmp plate. PCR products so prepared were run on an automatic sequencer ABI PRISM 3100 DNA Sequencer (Applied Biosystem) and Analyzed with Gene Mapper Software® (Applied Biosystems, Foster City).

The size distribution of different TCR fragments was studied by utilizing a analysis' peak of CDR3 length. The resulting underloaded peaks were all ran again and when the software was not able to quantify any area under each peak we defined them as not detectable (nd).

TCRBV-CDR3 spectra were categorized into one of the three characteristics types of TCRBV-CDR3 length spectra: one consisting of a multipeak Gaussian-like pattern “bell shaped distribution” also called polyclonal (p); polyclonal-altered profiles (non-Gaussian distribution of peaks) indicating restricted T cell populations (pa); a skewed/perturbed shape consisting of 1-4 peaks or a multipeak pattern with one solitary peak >50% of the total area, and monoclonal pattern (SK) (Cancrini *et al.* 2005).

Our approach focuses on quantifying the percentage of altered family over the 24 analyzed families.

Materials:

- anti-CD4 or anti-CD8 monoclonal antibody-coupled magnetic beads (Dynal AS, Oslo, Norway)
- TRIzol® (Gibco-BRL/Life Technologies, Milan, Italy)
- SuperScript One-Step RT-PCR kit (Invitrogen, Milan, Italy)
- 5’Vb and 3' Cb primer (Mmedical, Milan, Italy)
- Hi-Di Formamide (Applied Biosystems)
- MicroAmp optical 96 well (Applied Biosystems)
- 5’Vb and 3' Cb primer (Mmedical, Milan, Italy)

Sequences of primers used were the followings:

TCRBV 1	5’ - CAG	TTC	CCT	GAC	TTG	CAC	TC – 3’	
TCRBV 2	5’ - GCT	TCT	ACA	TCT	GCA	GTG	C – 3’	
TCRBV 3	5’ - GAG	AGA	AGA	AGG	AGC	GCT	TC – 3’	
TCRBV 4	5’ - GCA	GCA	TAT	ATC	TCT	GCA	GC – 3’	
TCRBV 5.1	5’ - CTC	GGC	CCT	TTA	TCT	TTG	CG – 3’	
TCRBV 5.3	5’ - CCC	TAA	CTA	TAG	CTC	TGA	GC – 3’	
TCRBV 6.1	5’ - GAT	CCA	GCG	CAC	ACA	GC – 3’		
TCRBV 6.2	5’ - GAT	CCA	GCG	CAC	AGA	GC – 3’		
TCRBV 7	5’ - CCT	GAA	TGC	CCC	AAC	AGC – 3’		
TCRBV 8	5’ - GAA	CCC	AGG	GAC	TCA	GCT	G – 3’	
TCRBV 9	5’ - GGA	GCT	TGG	TGA	CTC	TGC	TG – 3’	
TCRBV 11	5’ - CAG	GCC	CTC	ACA	TAC	CTC	TCA – 3’	
TCRBV 12	5’ - CAA	AGA	CAG	AGG	ATT	TCC	TCC – 3’	
TCRBV 13	5’ - GTC	GGC	TGC	TCC	CTC	CC		
TCRBV 14	5’ - GTC	TCT	CGA	AAA	GAG	AAG	AAG – 3’	
TCRBV 15	5’ - GTC	TCT	CGA	CAG	GCA	CAG	GC – 3’	
TCRBV 16	5’ - GAA	CTG	GAG	GAT	TCT	GGA	GTT – 3’	
TCRBV 17	5’ - CCA	AAA	GAA	CCC	GAC	AGC	TTT	C – 3’
TCRBV 18	5’ - GTG	CGA	GGA	GAT	TCG	GCA	GC – 3’	
TCRBV 20	5’ - CCT	CCT	CAG	TGA	CTC	TGG	C – 3’	
TCRBV 21	5’ - GGC	TCA	AAG	GAG	TAG	ACT	CC – 3’	
TCRBV 22	5’ - GTT	GAA	AGG	CCT	GAT	GGA	TC – 3’	
TCRBV 23	5’ - CAG	TTC	AGT	GAC	TAT	CAT	TCT	G – 3’
TCRBV 24	5’ - GGG	GAC	GCA	GCC	ATG	TAC	C – 3’	
TCRBC FAM	5’ – TTC	TGA	TGG	CTC	AAA	CAC – 3’		

Cell staining and Flow cytometric analysis

Methods:

PBMCs were isolated from blood samples according to standard procedures. Cell surface staining was performed using different combinations of fluorochromes conjugated to monoclonal antibodies (MoAbs). Intracellular staining using anti-FoxP3 antibody was performed using a FoxP3 staining set (eBiosciences, San Diego, CA, USA) according to modified manufacturer's protocol.

Four-color FACS acquisition was performed on a FACSCalibur cytometer (Becton Dickinson, San Jose, California, USA) using CellQuest software (BD Biosciences). Optimal analysis required the acquisition of at least 200000 events within lymphocyte light-scatter gate. Data were analyzed using CellQuest software (Becton Dickinson, Mountain View, CA) and FlowJo software (TreeStar, Ashland, OR). Median of percentages and absolute count of CD3, CD3CD4 ($CD4^+$) and CD3CD8 ($CD8^+$) T-cells, naïve and memory T-cell indentified through the expression of CD45RA (naïve) and CD45RO (memory), $CD19^+$ B-cells and Natural Killer $CD16^+CD56^+$ (NK) cells subsets were compared with age-matched values (reference values from Shearer (Shearer *et al.* 2003)).

Moreover B-cell memory ($CD27^+$) and class-switched ($CD27^+IgD^-IgM^-$) and non-class switched ($CD27^+IgD^+IgM^+$)were analyzed in a more restricted number of them.

nTreg cell identified through $CD25^+CD127^{low/neg}FoxP3^+$ expression in $CD4^+$ lymphocytes as shown in Figure 10.

Materials:

The following antibodies were used:

- anti-Human CD127(IL7r)-PECy7 #25-1278-73 clone eBioRDR5 eBioscience
- anti-Human CD25-PE clone M-A251 #555432 BD Bioscience Pharmingen
- anti-Human CD27-PE clone MT271 BD Bioscience Pharmingen
- anti-Human CD4-APC clone RPA-T4 #555349 BD Bioscience Pharmingen
- anti-Human FoxP3 Staining Set #72-5776-40 eBioscience
- anti-Human FoxP3-Alexa Fluor 488 clone 259D #320212 Biolegend
- anti-Human IgD-FITC clone IA6-2 BD Bioscience Pharmingen
- anti-Human IgM-Cy5 #109-176-129 Jackson ImmunoResearch Laboratories
- BD Multitest CD3/CD16CD56/CD45/CD19 clone SK7/B73.1, NCAM16.2/2D1/SJ25C1 BD Bioscience Pharmingen
- BD Multitest CD45RA/CD45RO/CD3/CD4 clone L48/SK3/SK7/UCHL1 BD Bioscience Pharmingen
- BD Multitest CD45RA/CD45RO/CD3/CD8 clone L48/SK1/SK7/UCHL1 BD Bioscience Pharmingen

Statistical analysis

Immunologic parameters were not systematically studied; we analyzed immunological parameters that were available at every age. Lymphocytes subsets counts and percentages, lymphoproliferative SI values, TCRBV alteration values available for each patient were recorded in a worksheet (Excel; Microsoft Inc, Reddemond, Wash), and then the various data were processed using either SPSS software V.17 (SPSS, Chicago, Illinois, USA) or Prism 5 (GraphPad Software, San Diego, Calif). We divided the data regarding cohort

and age, considering the variability previously reported in infants with each pathology. The medians values of repeated immunological parameters for each time interval were calculated.

The Shapiro-Wilcoxon test was used to evaluate the Gaussian distribution of overall immunological parameters. Statistical comparisons between the different groups of patients and healthy control were then calculated with non-parametric analyses (Mann–Whitney non-parametric U-test) when no Gaussian distribution was found and exact p values were obtained, otherwise T-students' test was used. A significative threshold of $p<0.05$ was used to assess the statistical differences in patients versus healthy control. Results are reported either as the median and the 10^{th} and 90^{th} percentile {median [10^{th} – 90^{th}]}, or as the median and the 25^{th} and 75^{th} percentile {median [25^{th} – 75^{th}]}, or mean percentage and the standard deviation {mean, SD}.

RESULTS

Flow cytometric analysis

Flow-cytometric analysis was performed in 30 DiGeorge Syndrome (DGS), 12 Chronic Granulomatous Disease (CGD) and 24 Hypogammaglobulinemic (Hypogamma) patients.

In pDGS patients the percentages and counts of $CD3^+$ as $CD4^+$ and $CD8^+$ T-cell were decreased, although T-cell counts increased with the age as shown in Figure 1-3 (a, b).

No relevant differences in $CD3^+$ values such as $CD4^+$ and $CD8^+$ T-cells of CGD and Hypogamma patients were observed (Table 1-3) (Figure 1-3).

Interestingly, an imbalance toward memory phenotype for both $CD4^+$ and $CD8^+$ T-cell in each group of patients was observed (Table 4-7) (Figure 4 a, c; 5 a, c). Naïve T-cell (both $CD4^+$ and $CD8^+$) value in pDGS patients was marked reduced; this reduction was relevant in the first years of age although it persisted less evident during the follow-up (Table 5, 7) (Figure 4 d, 5 d). Naïve T-cell counts of CGD and Hypogamma patients were slightly reduced when compared with age-matched values (Table 4, 6) (Figure 4 b, 5 b).

No relevant NK and B cells values alteration in all patients was observed, although in pDGS patients a relative increase of NK and B cell frequencies was observed due to low level of T-cells (Table 8-9) (Figure 6-7).

Analysis of B-cell maturation in 25 pDGS and 6 CGD patients revealed a B-cell memory significative reduction in both class-switched and non-class switched B-cell memory compartment, when compared with 34 age matched healthy donors (Figure 8 a, b, c).

No correlation was found between low T-cell values and recurrent infections while a correlation between reduced $CD27^{+}$ B-cell memory and recurrent infections ($p<0.001$) was observed. Indeed 64% of pDGS patients with low B-cell memory values had recurrent infections while only 9.5% of patients with normal B-cell memory values had recurrent infections ($p<0.001$) (Figure 9). Noteworthy, all of pDGS patients with low B-cell memory have TCRBV alterations.

Natural regulatory T-cell

A total of 14 DiGeorge, 10 CGD, and 24 Hypogamma patients were analyzed for the percentages of nTreg subpopulation compared to 14 healthy controls. The mean percentage of $CD25^{+}CD127^{low/neg}FoxP3^{+}$ T cells expressed as percentage of $CD4^{+}$ lymphocytes when compared with healthy control value in each cohort of patients didn't show statistical differences (Figure 11).

Particularly in the unique pDGS patient with autoimmune manifestation a higher frequency of nTreg marker among the pDGS patients group was observed.

Lymphoproliferation assay

We studied the lymphoproliferative response of 30 DGS, 11 CGD and 22 Hypogamma patients compared to 270 healthy controls. Medians and [25^{th} - 75^{th}] percentile ranges of PBMC proliferation SI at different ages are shown in Figure 13-15 below.

A statistical significant reduced lymphoproliferative responses to stimuli (PHA, OKT3 and PWM) were found in each cohort of patients (Figure 15-17 a, b) when compared with our age matched healthy controls (Table 10-12).

In pDGS patients a transient improvement was observed in the group between 2 and 6 years of age, for the all three stimulation.

During the follow-up in pDGS patients an improvement of proliferative response to all stimula was observed. Noteworthy, CGD patients showed an unexpected marked reduced proliferative response to PHA and OKT3.

TCRBV repertoire

CDR3 length distribution of 24 TCRBV families was analyzed in $CD4^+$ and $CD8^+$ T-cell by spectratyping in 14 DGS, 11 CGD, 13 Hypogamma and 20 newborn CMV congenital infected patients and 8 healthy donors.

TCRBV alterations were observed in all three patients' groups and appear with a higher degree in $CD8^+$ T-cell subset than in $CD4^+$ T-cell subset as shown in Figure 12 a (CD8 DGS mean: 15.2, SD=21.8; CGD mean: 23.8, SD=22.2; Hypogamma mean: 16, SD=23.4 vs

CTR mean: 2.2 ± 6.4) (CD4 DGS mean: 3.2, SD=4.3; CGD mean: 5.8, SD=9.3; Hypogamma mean: 2.1, SD=4.3 vs healthy donors mean: 2.1, SD=4.6) (Figure 12 a).

TCRBV family distributions in pDGS patients showed a trend of normalization both in $CD4^+$ and $CD8^+$ T-cell subsets during the follow-up as shown in Figure 14.

As already reported $CD4^+$ TCRBV family alterations were fewer than in $CD8^+$ subset. Only three pDGS patients (P4, P5 and P11) showed some alterations in some TCRBV families (8.6, 13.6 and 20% respectively) (Figure 14 a, b) which normalize sooner and better than $CD8^+$ TCRBV repertoire during the follow-up. In the congenital CMV infected newborn, *analyzed as a control group,* no TCRBV alterations were observed in CD4 subset either at the beginning or at the end of the follow up, showing normal polyclonal profiles of the TCR repertoire. On the other hand relevant TCRBV alterations in $CD8^+$ T-cells normalizing during the follow-up were found (Figure 12 b).

Noteworthy alterations in TCRBV family distribution were present mainly in pDGS patients with recurrent infections (*ri*) (Figure 13). Recurrent infections correlate with a high frequency of TCRBV alteration in both $CD4^+$ and $CD8^+$ T-cell subsets. Indeed $CD4^+$ TCRBV repertoire alterations, in patients with recurrent infections, were increased 3 fold more than patients without recurrent infections (*wri*) (*ri* $CD4^+$ mean: 5.6 ± 5.1; *wri* $CD4^+$ mean: 1.7 ± 2.3; p=0.01) (Figure 13 a). $CD8^+$ TCRBV repertoire showed better this difference,

indeed in *ri* patients TCRBV repertoire was 8,8 fold and statistical significative altered than in *wri* patients (*ri* $CD8^+$ mean: 30.1 ± 24.8; *wri* $CD8^+$ mean: 3.4 ± 3.5; p=0.04) (Figure 13 b).

DISCUSSION

Our data confirm that in pDGS patients the immunodeficiency is mild, more evident in the first years of age and often improving over time in some of them. In agreement with previous report we detected frequent and severe perturbations of TCRBV families principally on peripheral blood $CD8^+$ cells, moreover we detected less frequent but significative alterations in the $CD4^+$ subset (Sullivan *et al.* 1999). A known, immunocompetent individual maintain a wide diversity of TCR repertoire. Alterations, found in particular in TCRBV $CD8^+$ T cells in DGS, might reflect a diminished cellular turn-over of this cell population as occurs in patients with congenital/acquired athymia (Masci *et al.* 1999; Pignata *et al.* 2001), those underwent to chemotherapy (Mackall 1999) or affected by chronic infection (Romiti *et al.* 2001).

Since in DGS patients the altered TCR patterns were detected in the early samples, before the development of significant infections, the possibility that restriction of the T-cell receptor in $CD8^+$ T cells is merely the result of antigenic stimulation as reported in chronic infection or observed in congenital CMV infants (control group) is unlikely. It is likely that the altered TCR repertoire of $CD8^+$ T-cells in children with pDGS reflects a developmental defect rather than an antigenic stimulation. This hypothesis is supported by the absence of alterations in TCRBV family distribution in patients with humoral defect and in X-linked agammaglobulinaemia (XLA) patients

analyzed, suffering of recurrent infections and by the demonstration of a decreased thymic output by the reduction of TREC (Pierdominici *et al.* 2003) in these patients.

Lymphopenia found in these patients, might be compensated to peripheral extra-thymic reconstitution as suggested by naïve and memory T-cell percentages that in our cohort showed an imbalance towards memory phenotype in both $CD4^+$ and $CD8^+$ T-cell subsets (Piliero *et al.* 2004). This process could not provide a complete immunoreconstitution as occurred in congenital CMV infection or in some HAART HIV infected infants where an efficient thymic function occurred (Romiti *et al.* 2001) indeed, slow but not complete normalization of TCRBV distribution was observed during follow-up.

Furthermore, since a widely diverse polyclonal TCR repertoire is indicative of an intact T-cell population with the potential to recognize a wide range of immunogens, restriction in the TCR repertoire and oligoclonal expansions are associated to an increased risk of developing infections and/or autoimmunity (Gras *et al.* 2008). It is likely that patients with marked alterations in TCR repertoire distribution are at a greater risk to develop recurrent infections. Indeed, we observed that among DGS patients, those who presented a higher frequency of early TCRBV abnormalities were also those that suffered recurrent infections: *ri*-DGS patients showed higher alterations than *wri* patients at the baseline.

Finally the risk of developing autoimmunity seems to be contingent to an altered T cell development as these patients show oligoclonal alterations of TCR repertoire related to an altered thymic output. Indeed, the unique our DGS patient with autoimmunity showed an initial widely altered TCR repertoire, as patients that suffer of recurrent infections.

Although the immunodeficiency of DGS is classically described as cell-mediated, alterations in the humoral compartment, not clearly demonstrated as related to T-cell deficiency or to intrinsic B-compartment defect, were reported by our and other groups (Finocchi *et al.* 2006). Indeed, extending our previous study, analysis of both non-class and class switched memory B-cell showed relevant correlations with recurrent infections. These data, in association with the clinical/immunological features of *ri* patients, suggest that defects in B-cell compartment could be associated to a higher risk of infections in DGS patients so to represent another prognostic marker. Indeed, we observed that the patients with humoral abnormalities showed a severely restricted TCR repertoire. One could speculate that alterations in T-cell compartment may have an effect on humoral immune function in this group of patients. Interestingly, the human IgL locus is localized at 22q11.2 chromosome (Lefranc 2001). Then, it is still unknown if the B-cell defect is primitive or caused by altered T-cells.

Finally, nTreg cell compartment in our patients did not confirm previous data on Treg, where a decrease in DGS patients was observed (Sullivan *et al.* 2002; McLean-Tooke *et al.* 2008). Indeed, in the unique our patient with autoimmune disease, nTreg percentage was the higher among the DGS patients. Differences in the methods used to evaluate nTreg subpopulation may account for the observed discrepancies, although in tune with current scientific opinion at the age of the study.

Alternatively a different interpretation should be sought in line with the hypothesis of Bacchetta et al. (Bacchetta *et al.* 2006), who suggested that human IPEX is not necessarily due to the absence of Treg cells but rather to their impaired suppressive function. Further functional studies are needed to establish the actual role of these cells in our cohort of patients.

Although CGD immunodeficiency is classically described as disorder caused by inherited defects in the NADPH oxidase complex used by phagocytic cells, we detected alterations both in B and T cell compartment of these patients. A marked reduction of memory B-cells was observed, both in class switched and non-class switched, confirming previously results where a role of ROS as crucial element of memory B cells maturation was hypothesized (Bleesing *et al.* 2006). Interesting, extensive analysis of T-cell compartment revealed the reduction of naïve T cells with expansion of memory

$CD45RO^{+}CD45RA^{-}$ phenotype and a reduced proliferation capacity upon stimulation, as already reported by (Heltzer *et al.* 2002). The progressive depletion of naïve T cells and the expansions of TCRBV families, mostly in $CD8^{+}$ T-cells of CGD patients could be due to chronic antigenic stimulation resulting in expansion of specific memory cells. Alternatively, the observed T cell alterations may be due to apoptotic abnormalities not completely compensated by a recovering of naïve T-cell. Indeed recently has been demonstrated that NADPH oxidase system beside B-cell might play a role in T-cell subsets (Jackson *et al.* 2004). Mainly reactive oxygen species derived from T-cells' NADPH oxidase appears to have an anti-inflammatory activity, ROS can affect this activity by sensitizing T-cell to apoptosis (Tripathi and Hildeman 2004; Munder *et al.* 2006; Schappi *et al.* 2008b; Purushothaman *et al.* 2009).

Our data showed that quantitative and qualitative alterations are common in T-cells of CGD patients and might contribute to the heterogeneity of the clinical phenotype.

CONCLUDING REMARKS

The result of our study could contribute to improve our understanding (contribute to explain) of the heterogeneity of the clinical and the immunological phenotype of these patients. Indeed, alterations of T cell repertoire distribution and B cell maturation observed in some patients, could play an important role in the pathogenesis of the immunological defect and explain the extent of immunodeficiency. Immunological alterations variability and patients' clinical expression does not allow easily to make standard protocol of diagnosis and therapy in these patients, therefore some parameters such as TCRBV family distribution and B-cell maturation could be used as further prognostic markers in order to provide useful information to play an appropriate, personalized, follow-up and specific treatment.

Further studies in a larger number of patients are needed to identify, first which immunological parameters influence the development of infections and/or autoimmunity in these patients, and second to clarify if the B-cell defect is intrinsic or related to the T-cell defect.

TABLES AND FIGURES

Table 1: CD3^{+} T cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD3+ T cells (%)	CTR (Shearer et al. 2003)	73 [53-84]	66 [51-77]	65 [49-76]	65 [53-75]	66 [56-75]	69 [60-76]
	Di George	**46.5** [34-69] n=6	**43** [31.9-65] n=9	**40.1** [31.2-66] n=8	**33.5** [26-60] n=7	**47.8** [35.6-66.2] n=20	**54.2** [41.5-63.5] n=9
	CGD	- -	- -	69 n=1	68.4 n=1	65 [58-73] n=4	73 [57.9-78] n=4
	Hypogamma	- -	- -	- -	- -	71.43 [69-89] n=6	71.9 [54-88.9] n=20
CD3+ T cells (cells/µl)	CTR (Shearer et al. 2003)	3680 [2500-5500]	3930 [2500-5600]	3930 [1900-5900]	3550 [2100-6200]	2390 [1400-3700]	1820 [1200-2600]
	Di George	**1458** [450-3064] n=4	**1394** [925-1875] n=3	**1252** [748-1785] n=7	**1372** [1134-2223] n=6	**920** [745-1995] n=15	**912** [499-1367] n=8
	CGD	- -	- -	2636 n=1	3365 n=1	1985 [1201-3395] n=4	1697 [1069-2215] n=4
	Hypogamma	- -	- -	- -	- -	2739 [2452-2961] n=5	1787 [801-2440] n=20

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 2: CD4$^+$ T cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD4+ T cells (%)	CTR (Shearer et al. 2003)	52 [35-64]	46 [35-56]	46 [31-56]	41 [32-51]	38 [28-47]	37 [31-47]
	Di George	35.5 [23.3-52] n=6	**30** [23-42] n=9	**25.7** [21-37.7] n=8	**20** [17-45] n=7	27.9 [20-44.3] n=20	**28 [22-37.5] n=9**
	CGD	- -	- -	51 n=1	48 n=1	29.5 [22-41] n=4	38 [31.4-41] n=4
	Hypogamma	- -	- -	- -	- -	42.9 [29-50.6] n=6	42.7 [26.3-55.3] n=20
CD4+ T cells (cells/µl)	CTR (Shearer et al. 2003)	2610 [1600-4000]	2850 [1800-400]	2670 [1400-4300]	2160 [1300-3400]	1380 [700-2200]	980 [650-1500]
	Di George	**1058** [294-2309] n=4	**1082** [650-1221] n=3	**893** [512-1369] n=7	**919** [528-1002] n=6	**547** [380-1068] n=15	**450** [295-811] n=8
	CGD	- -	- -	1948 n=1	2322 n=1	889 [693-1130] n=4	928 [521-1079] n=4
	Hypogamma	- -	- -	- -	- -	1403 [1247-2032] n=5	941 [487-1466] n=20

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 3: CD8$^+$ T cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD8+ T cells (%)	CTR (Shearer et al. 2003)	18 [12-28]	16 [12-23]	17 [12-24]	20 [14-30]	23 [16-30]	25 [18-35]
	Di George	12.8 [5-23] n=6	**9** [5.8-23] n=9	**8.9** [5.8-28] n=8	**9** [6-21] n=7	**12** [8-23.4] n=20	**16** [11.5-34] n=9
	CGD	- -	- -	13 n=1	16 n=1	25.4 [16-40] n=4	28 [20.5-31] n=4
	Hypogamma	- -	- -	- -	- -	23 [2-36] n=6	21.1 [13.8-34.8] n=20
CD8+ T cells (cells/µl)	CTR (Shearer et al. 2003)	980 [560-1700]	1050 [590-1600]	1040 [500-1700]	1040 [620-2000]	840 [490-1300]	680 [370-1100]
	Di George	**404** [95-755] n=4	**210** [200-371] n=3	**291** [197-320] n=7	**433** [170-1037] n=6	**276** [207-726] n=15	**291** [134-439] n=8
	CGD	- -	- -	497 n=1	818 n=1	617 [441-1968] n=4	608 [446-908] n=4
	Hypogamma	- -	- -	- -	- -	905 [763-1136] n=5	617 [182-959] n=20

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 4: Naïve CD4$^+$ T cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD4+CD45RA+ T cells (%)	CTR (Shearer et al. 2003)	90 [64-95]	90 [77-94]	86 [64-93]	81 [63-91]	71 [53-86]	59 [46-77]
	Di George	81.2 [59.4-91.3] n=5	70 [68.5-82.1] n=3	**60.1** [20.2-84.1] n=5	**52.5** [36.7-75.3] n=5	**60.6** [39.3-76.1] n=16	59 [32-76.2] n=8
	CGD	- -	- -	- -	**91.8** n=1	66.6 n=1	**64** [59.1-64.1] n=3
	Hypogamma	- -	- -	- -	- -	**66.7** [43.5-89.9] n=2	67.6 [10.5-78.4] n=14
CD4+CD45RA+ cells (cells/µl)	CTR (Shearer et al. 2003)	2270 [1200-3700]	2320 [1300-2700]	2210 [1100-3700]	1650 [1000-2900]	980 [430-1500]	570 [320-1000]
	Di George	**861 [174-1732] n=4**	**907** [811-1003] n=2	**486 [180-1104] n=5**	**350** [230-527] n=4	**370 [219-653] n=13**	**259 [136-443] n=7**
	CGD	- -	- -	- -	1719 n=1	**407** n=1	**469** [373-586] n=3
	Hypogamma	- -	- -	- -	- -	690 n=1	**589** [102-1053] n=14

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 5: Memory CD4$^+$ T cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD4+CD45RO+ T cells (%)	CTR (Shearer et al. 2003)	10 [2-22]	8 [3-16]	9 [5-18]	12 [7-20]	16 [9-26]	21 [13-30]
	Di George	18.7 [8.6-40.5] n=5	**30** [17.8-31.4] n=3	**39.9** [15.8-79.7] n=5	**47.4** [24.6-64.2] n=5	**39.3** [28.8-60.6] n=16	**40.9** [23.7-68] n=8
	CGD	- -	- -	- -	8.1 n=1	**33.3** n=1	**35.9** [35.8-40.8] n=3
	Hypogamma	- -	- -	- -	- -	**33.2** [10-56.4] n=2	**32.3** [21.5-89.4] n=14
CD4+CD45RO+ T cells (cells/µl)	CTR (Shearer et al. 2003)	320 [60-900]	330 [120-630]	340 [160-800]	400 [210-850]	360 [220-660]	350 [230-630]
	Di George	168 [92-400] n=4	295 [218-373] n=2	350 [139-711] n=5	377 [169-424] n=4	230 [125-643] n=13	**194** [158-262] n=7
	CGD	- -	- -	- -	**153** n=1	**203** n=1	267 [209-404] n=3
	Hypogamma	- -	- -	- -	- -	**894** n=1	**298** [164-480] n=14

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 6: Naïve CD8$^+$ T cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD8+CD45RA+ T cells (%)	CTR (Shearer et al. 2003)	93 [80-99]	94 [85-98]	91 [75-97]	89 [71-98]	86 [69-97]	80 [64-92]
	Di George	93 [75.6-99.2] n=5	85.2 [82.2-92.9] n=3	**68.4** [5-90.5] n=5	79.1 [13.1-87.9] n=5	75.1 [39.1-85.8] n=16	70.2 [41.6-92.8] n=8
	CGD	- -	- -	- -	92.3 n=1	84.6 n=1	**55.8** [55.5-85] n=3
	Hypogamma	- -	- -	- -	- -	52.4 [15-89.8] n=2	70.5 [47.6-88.8] n=14
CD8+CD45RA+ T cells (cells/µl)	CTR (Shearer et al. 2003)	870 [450-1500]	910 [550-1400]	870 [480-1500]	940 [490-1700]	670 [380-1100]	540 [310-900]
	Di George	494 [106-1176] n=4	**402** [344-460] n=2	**291** [14-649] n=5	**333** [169-790] n=4	**213** [125-521] n=13	**208** [80-285] n=7
	CGD	- -	- -	- -	**458** n=1	**373** n=1	**298** [296-416] n=3
	Hypogamma	- -	- -	- -	- -	**56** n=1	359 [155-570] n=14

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 7: Memory CD8$^+$ T cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD8+CD45RO+ T cells (%)	CTR (Shearer et al. 2003)	3 [1-9]	3 [1-7]	3 [1-8]	6 [2-12]	9 [4-16]	12 [4-21]
	Di George	6.9 [0.7-24.3] n=5	**14.7** [7-17.7] n=3	**31.5** [5.4-95] n=5	**20.8** [12-86.8] n=5	**24.8** [14.1-60.8] n=16	**29.7** [7.1-58.3] n=8
	CGD	- -	- -	- -	7.6 n=1	15.3 n=1	**44.1** [14.9-44.4] n=3
	Hypogamma	- -	- -	- -	- -	**47.7** [10.1-84.9] n=2	**29.4** [11.1-52.3] n=14
CD8+CD45RO+ T cells (cells/µl)	CTR (Shearer et al. 2003)	100 [30-330]	120 [30-290]	120 [40-330]	230 [60-570]	190 [90-440]	210 [70-390]
	Di George	38 [3-133] n=4	53 [26-80] n=2	95 [17-498] n=5	337 [47-1554] n=4	**76** [30-337] n=13	**68** [30-146] n=7
	CGD	- -	- -	- -	**38** n=1	**68** n=1	234 [72-238] n=3
	Hypogamma	- -	- -	- -	- -	317 n=1	132 [49-344] n=14

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 8: Natural Killer cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
NK cells (%)	CTR (Shearer et al. 2003)	8 [4-18]	6 [3-14]	7 [3-15]	7 [3-15]	9 [4-17]	9 [3-22]
	Di George	12.5 [5-22] n=4	**24.6** [10-64] n=8	**18** [6.8-31.2] n=7	20 [4-38] n=6	**16.1** [7.63-34] n=18	21.3 [11-28.2] n=8
	CGD	- -	- -	4 n=1	7 n=1	6.5 [3-17] n=4	4.6 [4.3-5.7] n=4
	Hypogamma	- -	- -	- -	- -	10.8 [2.5-14] n=5	9.7 [5-16] n=20
NK cells (cells/µl)	CTR (Shearer et al. 2003)	420,0	420,0	400,0	360,0	300,0	230,0
	Di George	419 [222-854] n=3	**912** [436-1600] n=3	**559** [161-1426] n=7	**1069** [76-1320] n=5	584 [159-920] n=14	349 [146-510] n=8
	CGD	- -	- -	153 n=1	405 n=1	202 [51-801] n=4	113 [84-172] n=4
	Hypogamma	- -	- -	- -	- -	461 [55-531] n=5	204 [88-527] n=20

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 9: B cells percentages and counts.

	Cohort	Age Group					
		0-3 mo	**3-6 mo**	**6-12 mo**	**1-2 yrs**	**2-6 yrs**	**6-12 yrs**
CD19 B cells (%)	CTR (Shearer et al. 2003)	15 [6-32]	25 [11-14]	24 [14-37]	25 [16-35]	21 [14-33]	18 [13-27]
	Di George	26.8 [9-34.3] n=6	28 [23-43] n=9	32.2 [24.7-42] n=7	34 [22-45.8] n=7	25.1 [20.3-32.4] n=18	20.8 [15.5-27.8] n=8
	CGD	- -	- -	22 n=1	20 n=1	22 [15-24.2] n=4	17.7 [12.2-31.7] n=4
	Hypogamma	- -	- -	- -	- -	13.7 [1.5-19.5] n=6	13.2 [2.1-28] n=20
CD19 B cells (cells/µl)	CTR (Shearer et al. 2003)	730 [300-2000]	1550 [430-300]	1520 [610-2600]	1310 [720-2600]	750 [390-1400]	480 [270-480]
	Di George	750 [398-1331] n=4	1216 [850-1875] n=3	1124 [621-1680] n=7	1109 [529-1927] n=6	676 [292-958] n=15	366 [195-546] n=8
	CGD	- -	- -	840 n=1	936 n=1	751 [254-1083] n=4	434 [182-1055] n=4
	Hypogamma	- -	- -	- -	- -	503 [50-680] n=5	301 [51-937] n=20

Values are presented as median and range [10th - 90th percentile]. Bold characters indicate medians outside normality range. In the grey box are represented both value and 10th - 90th percentile outside normality range.

Table 10: Lymphoproliferation upon PHA stimulation.

	Cohort	**Cohort-grouped**	Age Group			
			< 1 yr	**1-2 yrs**	**2-6 yrs**	**6-16 yrs**
SI PHA	CTR	70.2 [50.1-101.4] n=270	52.5 [39.9-77.2] n=36	74.2 [51.8-99.2] n=81	73.6 [50.9-127.3] n=54	70.6 [51.2-103.4] n=84
	Di George	**35.4** [24.9-62.2]** n=30	**25.3** [9.5-32.3]** n=10	**37.5** [20.7-88.7]** n=15	51.1 [34.5-94.4] n=11	**37** [28.8-68.7]* n=10
	CGD	**35.9** [21.4-47.6]** n=11	93 n=1	**19.7** [12-27.3]* n=2	**29.5** [28.9-30.2]* n=2	**41.3** [18.7-72.9]* n=6
	Hypogamma	**40.9** [20.2-69]** n=22	- -	**19,7** [13,9-53,8] n=4	**18.5** [10.7-51.1]* n=6	**41.2** [22.7-67.7]* n=19

Values are presented as median and range [25th - 75th]. Bold characters indicates median outside normality range. In the grey box are represented values with 25th and 75th outside normality range. * = ($p<0.05$), ** = ($p<0.01$).

Table 11: Lymphoproliferation upon OKT3 stimulation.

	Cohort	**Cohort-grouped**	Age Group			
			< 1 yr	**1-2 yrs**	**2-6 yrs**	**6-16 yrs**
SI OKT3	CTR	53.6 [34.8-78.6] n=270	38.2 [27.6-59.4] n=36	48.6 [34.4-71.8] n=81	58.7 [39.8-95.8] n=54	60.3 [35.9-86.3] n=84
	Di George	**25.7** [14-46.8]** n=29	**17.8** [2.9-30.1]** n=9	**31.4** [16.3-52.2]* n=15	46.8 [10.4-75.5] n=10	**16.9** [12.9-38.3]** n=10
	CGD	**33.9** [6-39.3]** n=11	69.3 n=1	**22.5** [8.5-36.7] n=2	44.6 [25.1-64.1] n=2	37.8 [5.8-40.8]* n=6
	Hypogamma	**28.2** [12.3-46.8]** n=22	- -	**12** [4.2-86.8] n=4	**26.5** [12.5-65] n=6	39.1 [19.7-63.7] n=19

Values are presented as median and range [25th - 75th]. Bold characters indicates median outside normality range. In the grey box are represented values with 25th and 75th outside normality range. * = ($p<0.05$), ** = ($p<0.01$).

Table 12: Lymphoproliferation upon PWM stimulation.

	Cohort	**Cohort-grouped**	Age Group			
			< 1 yr	**1-2 yrs**	**2-6 yrs**	**6-16 yrs**
SI PWM	CTR	59.4 [39.3-85] n=270	45.1 [32.5-69.2] n=36	60.1 [39.9-83.9] n=81	71.5 [46.4-104] n=54	58.8 [36.6-85.6] n=84
	Di George	**31** [16.7-56.4]* n=30	**20.8** [7-40]** n=10	**29.9** [16.7-36.8]** n=15	52.3 [18.5-79.5] n=10	**32** [13.2-38.6]** n=10
	CGD	50.9 [22.4-62.9] n=11	84.2 n=1	**20.8** [19.4-22.4]* n=2	62.4 [56.2-68.7] n=2	42.1 [20.6-53.7]* n=6
	Hypogamma	**35.5** [30-64.6]** n=22	- -	**20** [13.1-51.7] n=4	**17.6** [7.4-45]* n=6	**33.3** [21.6-63.1]* n=19

Values are presented as median and range [25th - 75th]. Bold characters indicates median outside normality range. In the grey box are represented values with 25th and 75th outside normality range. * = ($p<0.05$), ** = ($p<0.01$).

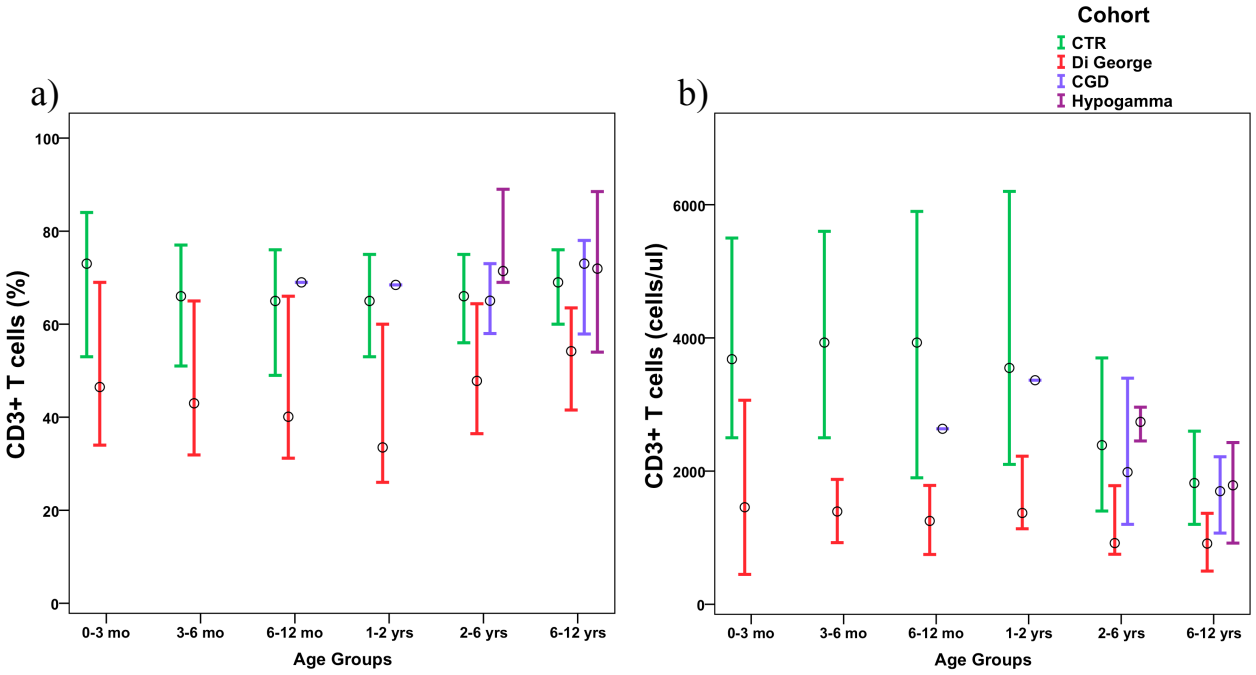


Figure 1: CD3$^+$ T cells percentage (a) and count (b). ○ of each bar indicates the median value, whiskers represent the 10th - 90th percentile. Look for the number in Table 1.

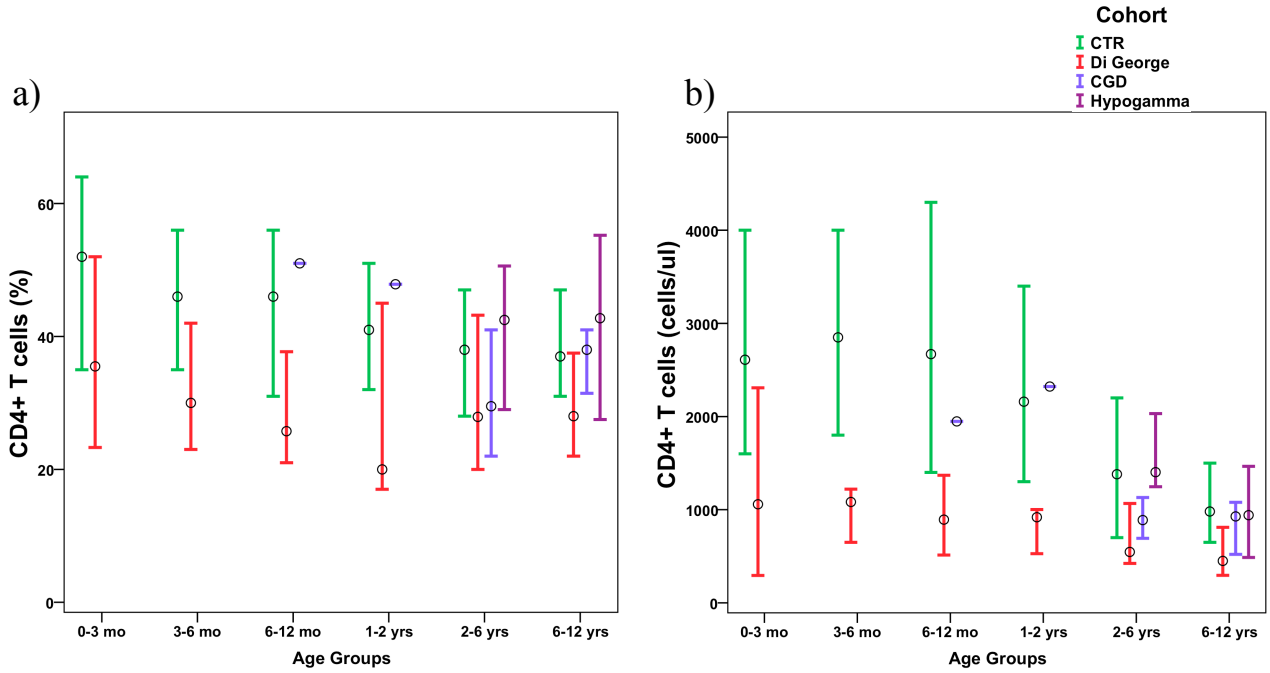


Figure 2: CD4^{+} T cells percentage (a) and count (b). ○ of each bar indicates the median value, whiskers represent the 10th - 90th percentile. Look for the number in Table 2.

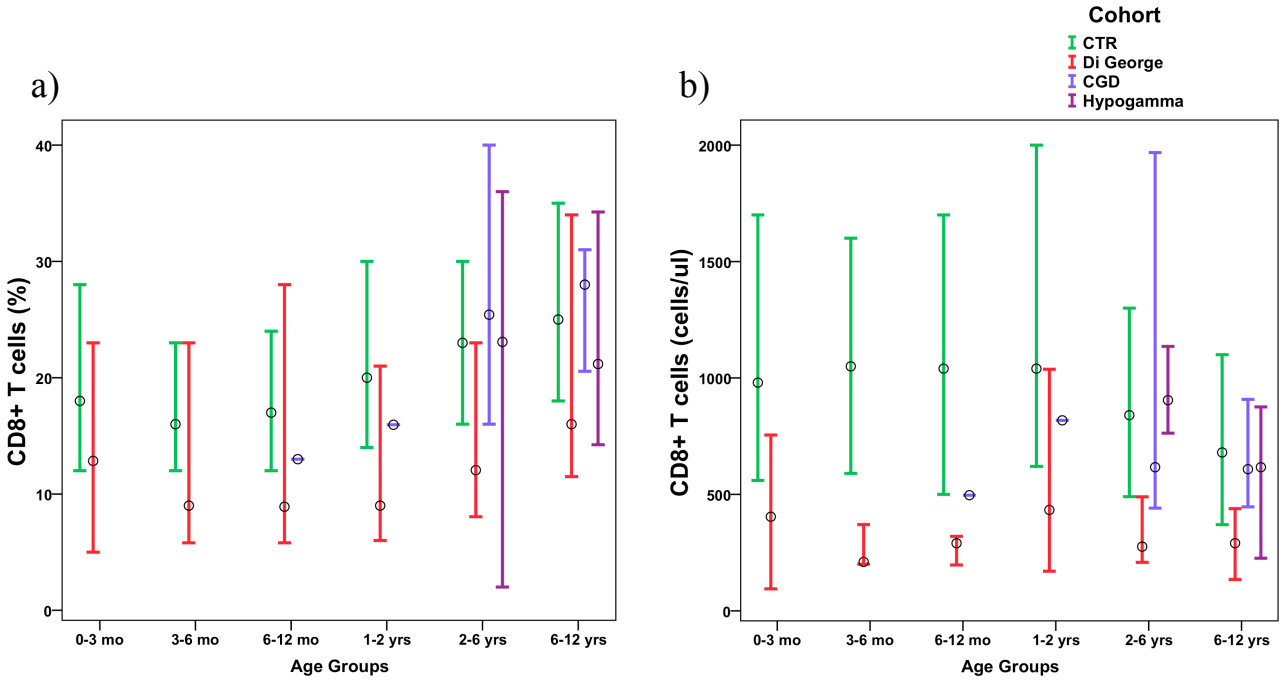


Figure 3: CD8$^+$ T cells percentage (a) and count (b). ○ of each bar indicates the median value, whiskers represent the 10th - 90th percentile. Look for the number in Table 3.

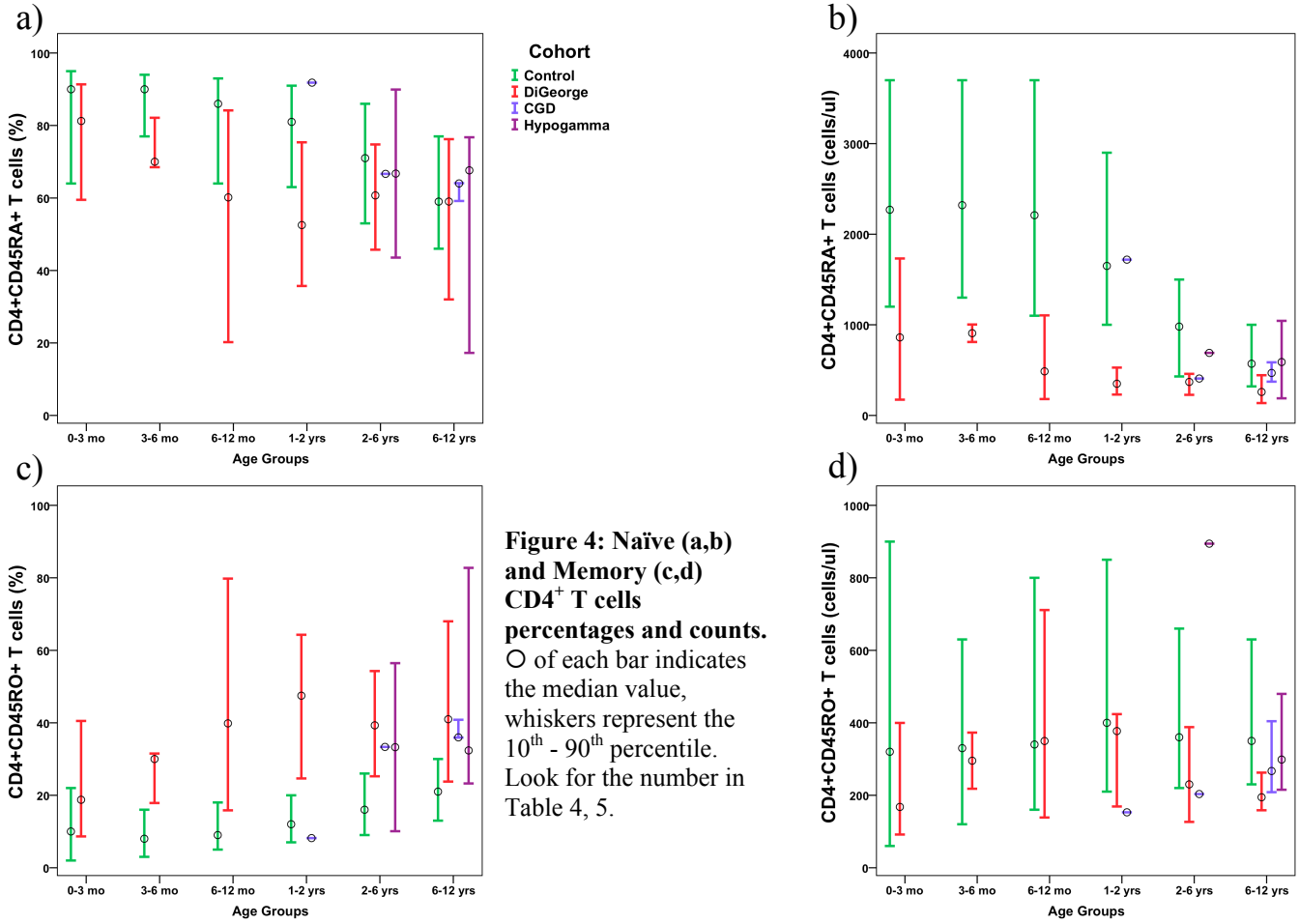


Figure 4: Naïve (a,b) and Memory (c,d) $CD4^+$ T cells percentages and counts. ○ of each bar indicates the median value, whiskers represent the 10^{th} - 90^{th} percentile. Look for the number in Table 4, 5.

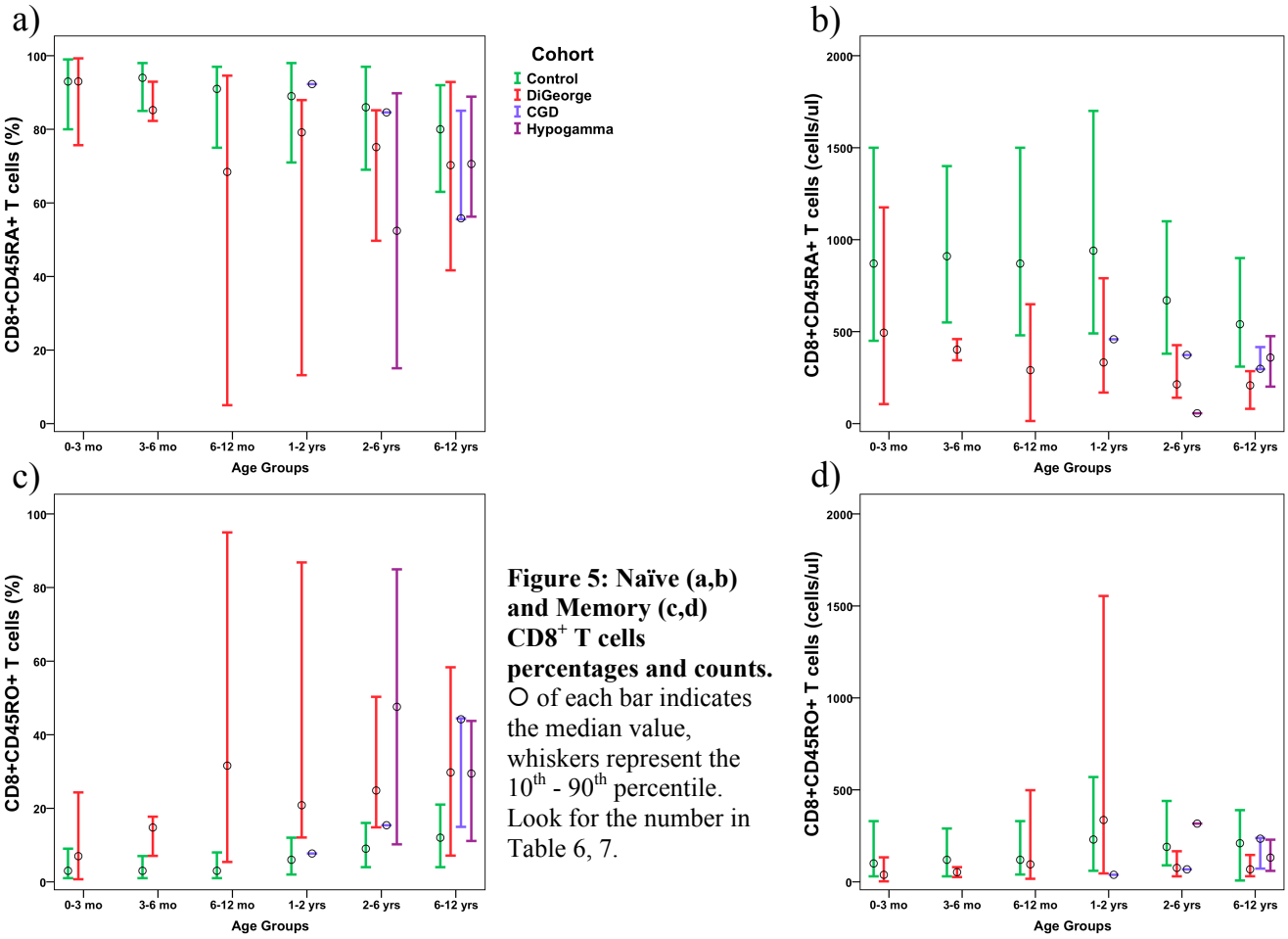


Figure 5: Naïve (a,b) and Memory (c,d) $CD8^+$ T cells percentages and counts. ○ of each bar indicates the median value, whiskers represent the 10^{th} - 90^{th} percentile. Look for the number in Table 6, 7.

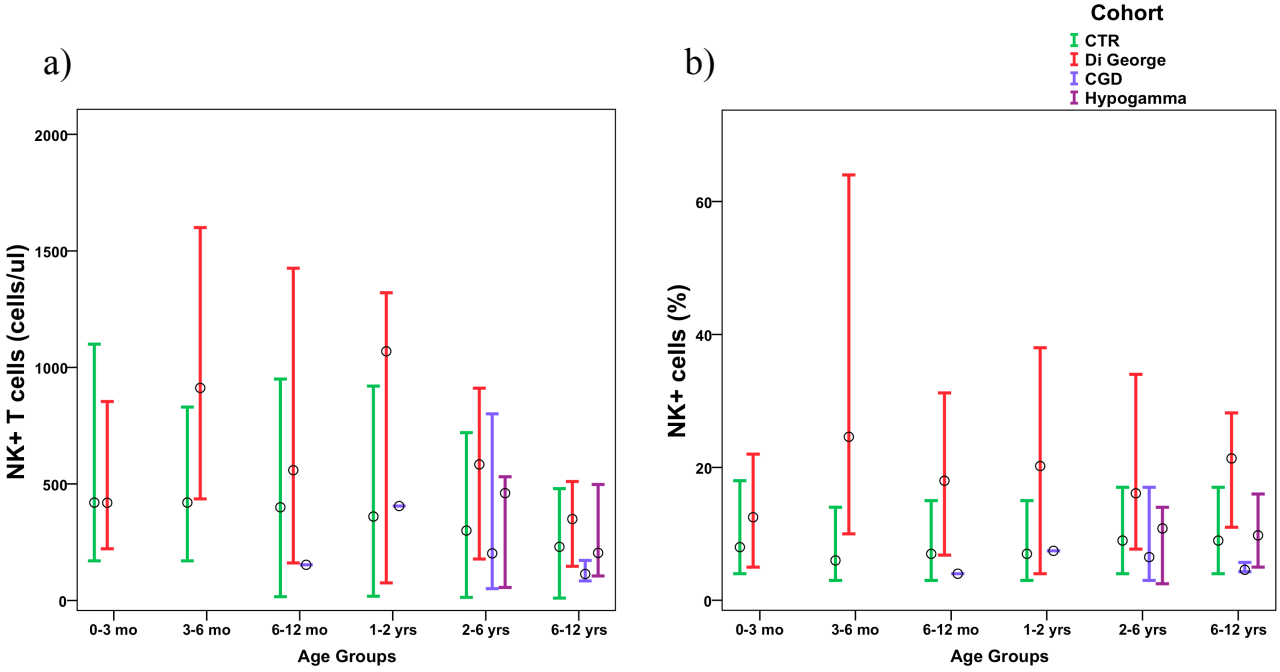


Figure 6: Natural Killer cells percentage (a) and count (b). ○ of each bar indicates the median value, whiskers represent the 10^{th} - 90^{th} percentile. Look for the number in Table 8.

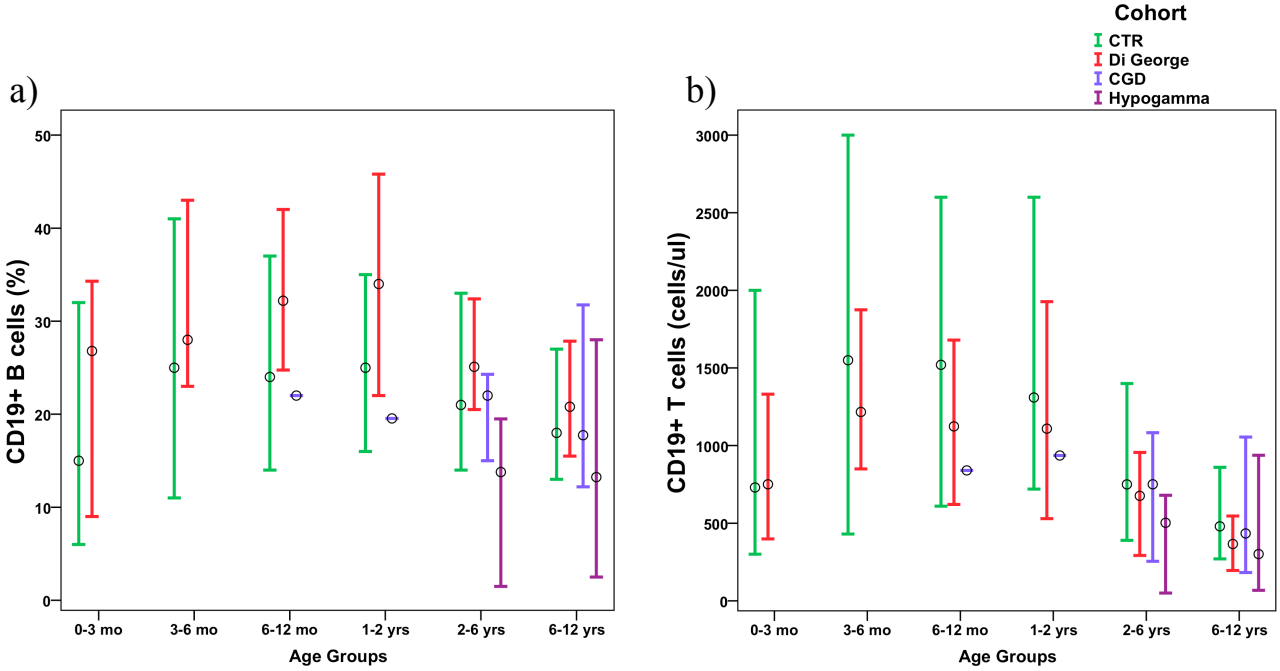


Figure 7: CD19^{+} B-cells percentage (a) and count (b). ○ of each bar indicates the median value, whiskers represent the 10th - 90th percentile. Look for the number in Table 9.

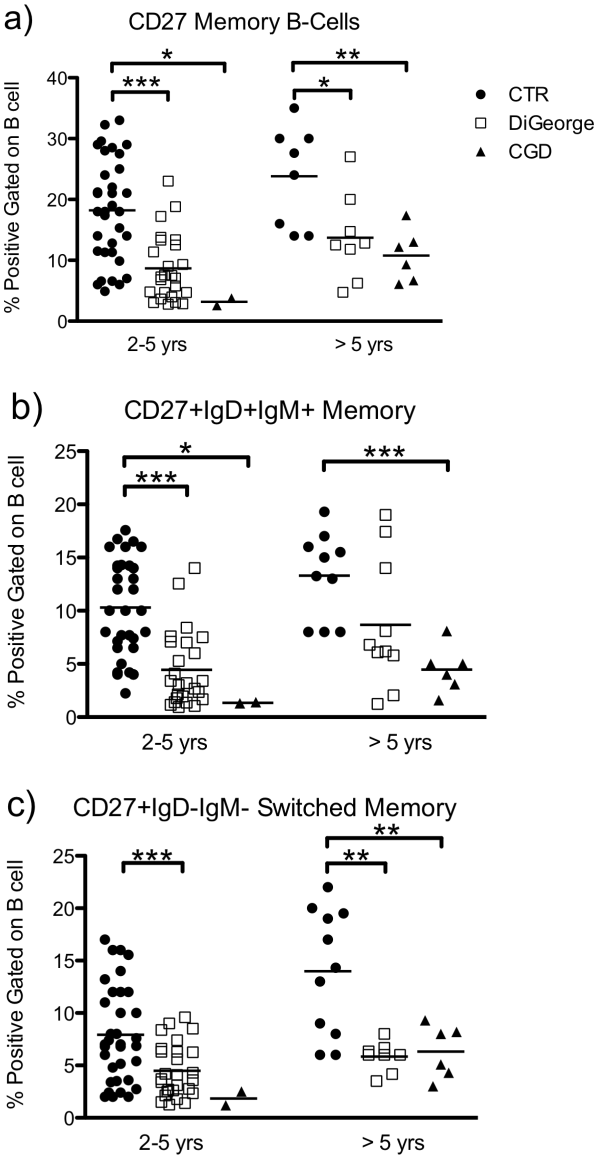


Figure 8: Memory B-cells subpopulation percentage. Each dot represents a different subject. $CD27^+$ Memory B-cells (a), non-class Switched $CD27^+$ Memory B-cells (b) and class Switched $CD27^+$ Memory B-cells (c) are shown. Horizontal bars indicate the mean value. p-values were determined by the Student's t-test: * = ($p<0.05$), ** = ($p<0.001$), *** = ($p<0.0001$).

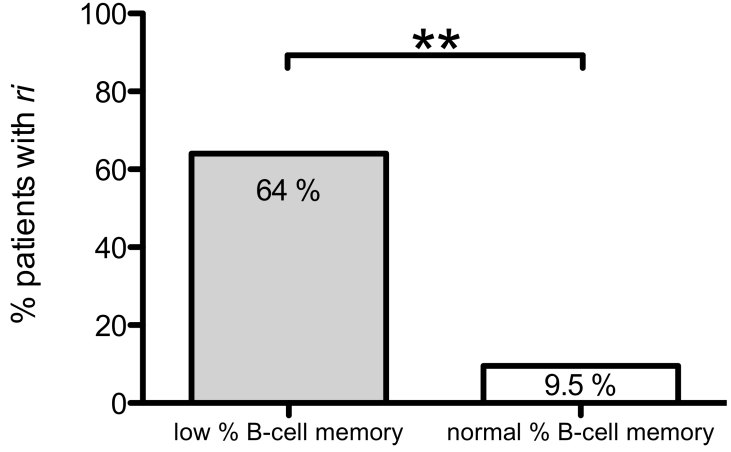


Figure 9: Occurence of recurrent infections (*ri*) in pDGS patients: patients with low $CD27^+$ B-cell memory (grey bar), patients with normal $CD27^+$ B-cell memory (white bar). ** = ($p<0.001$)

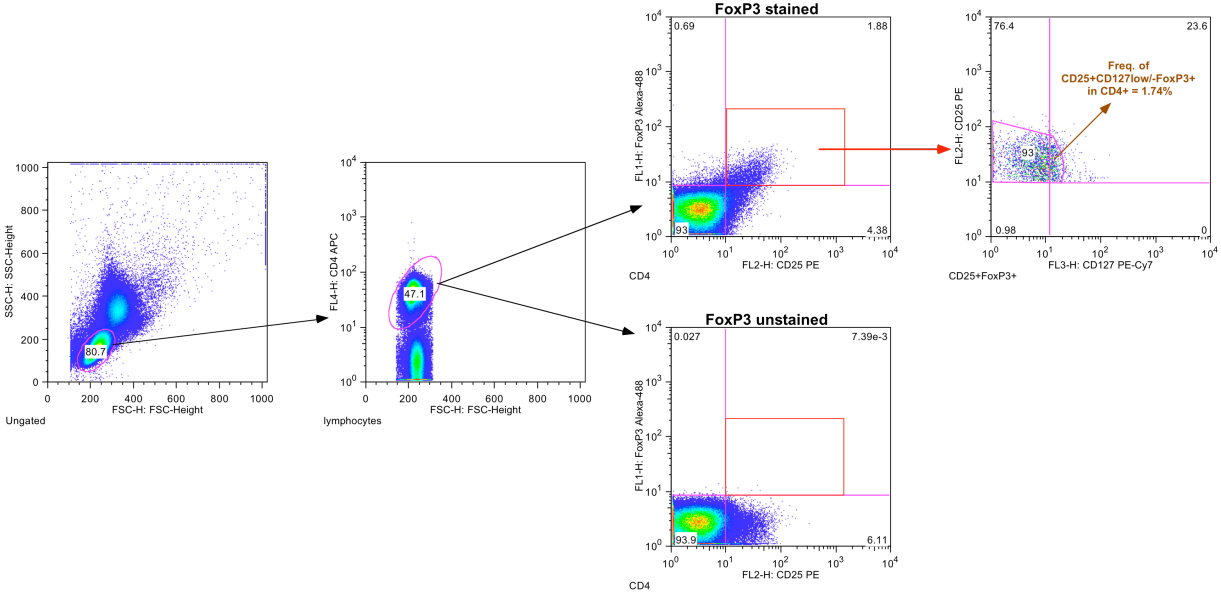


Figure 10: Gating strategy for nTreg subpopulations. According with the expression of CD4, CD25, CD127 and intracellular Foxp3, nTreg positivity was definited as FoxP3 in gated $CD25^{+}CD127^{low/-}$ lymphocytes.

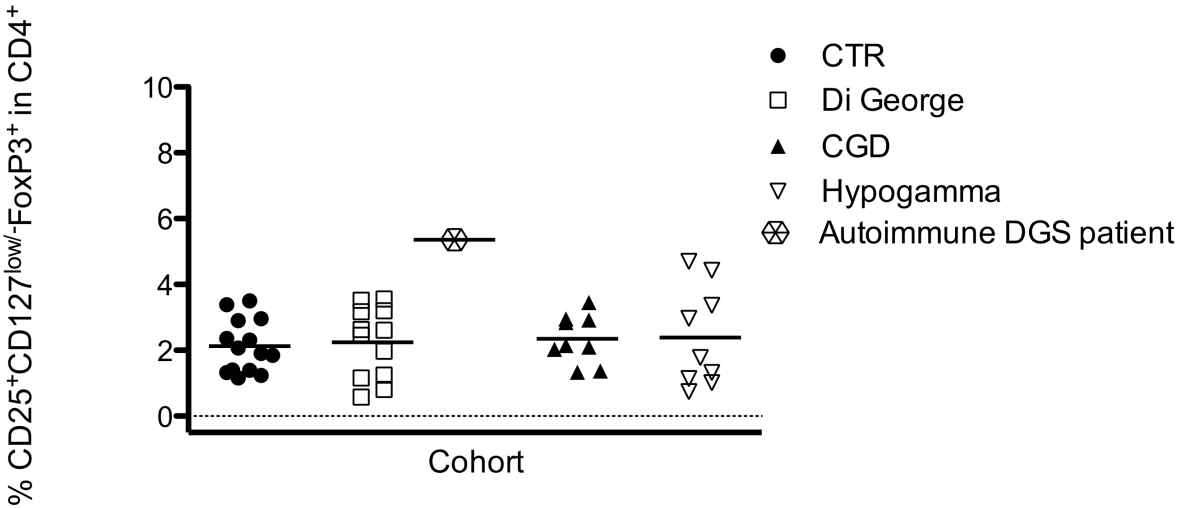


Figure 11: Frequency of $CD25^{+}CD127l^{ow/-}FoxP3^{+}$ on $CD4^{+}$ T-cell subset. Horizontal Bars represent mean value.

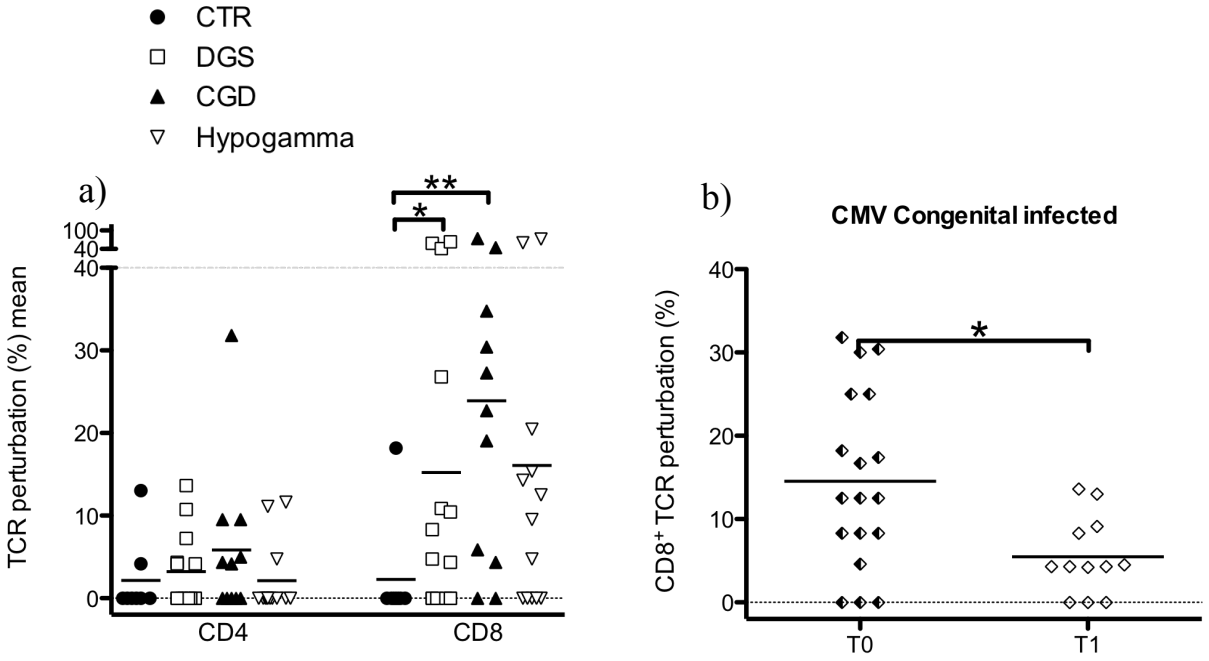


Figure 12: T cell receptor Vβ repertoire as measured by spectratyping of peripheral $CD4^+$ and $CD8^+$ T-cells. a) Cohort-grouped analysis, b) CMV congenital infected. Horizontal bars represent the mean values. p-values were determined by the Mann–Whitney U-test: * = ($p<0.05$), ** = ($p<0.001$).

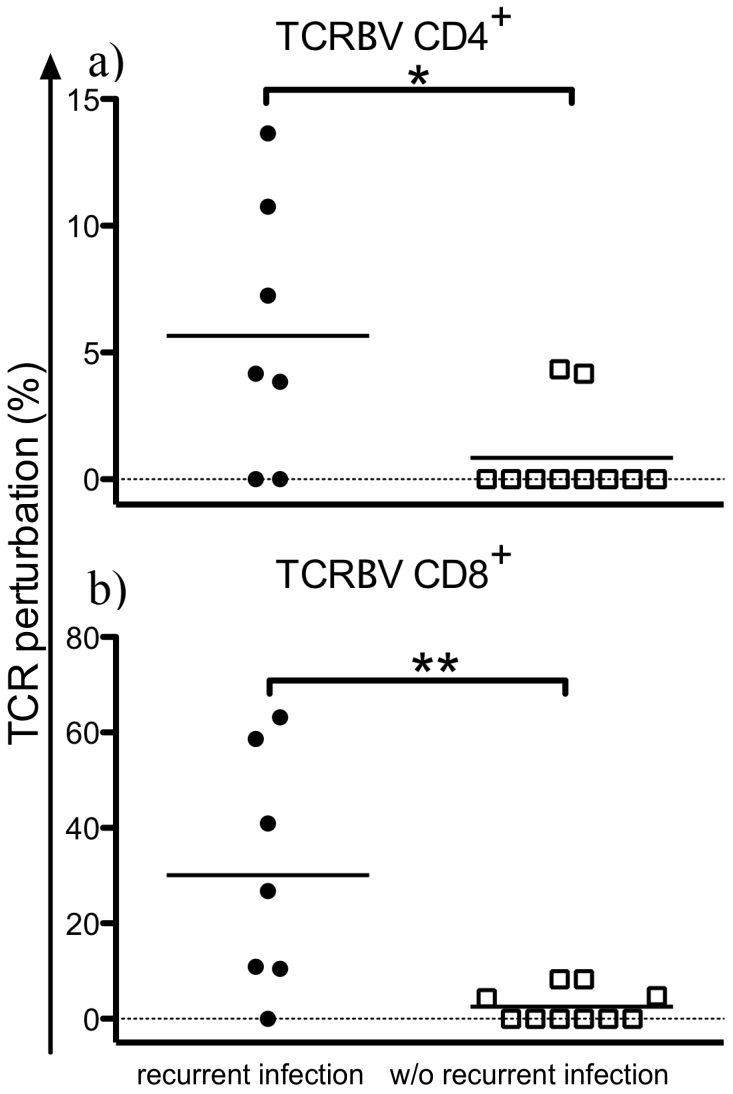


Figure 13: TCRBV perturbation in $CD4^+$ (a) and $CD8^+$ (b) pDGS patient with recurrent (circles) or without recurrent infections (squares). Horizontal bars represent mean values. p-values were determined by the Student's t-test: * = ($p<0.05$).

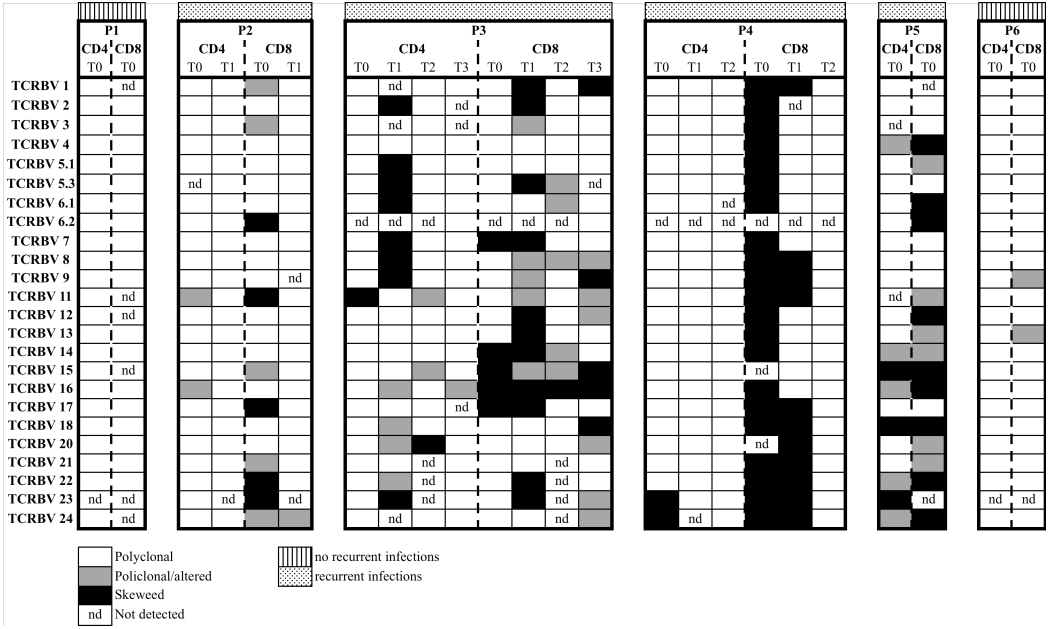


Figure 14 a: T cell receptor Vβ repertoire follow-up analysis in $CD4^+$ and $CD8^+$ T-cells in pDGS patients. Boxes represent single TCRBV families. See legend for details.

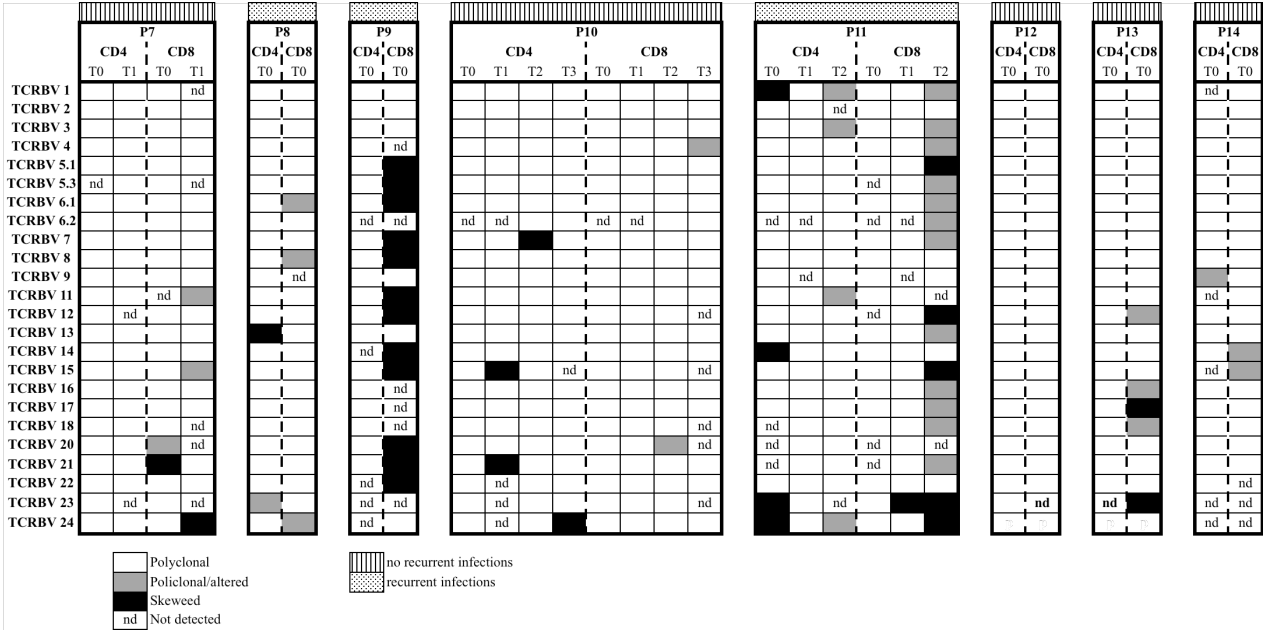


Figure 14 b: T cell receptor Vβ repertoire follow-up analysis in CD4$^+$ and CD8$^+$ T-cells in pDGS patients. Boxes represent single TCRBV families. See legend for details.

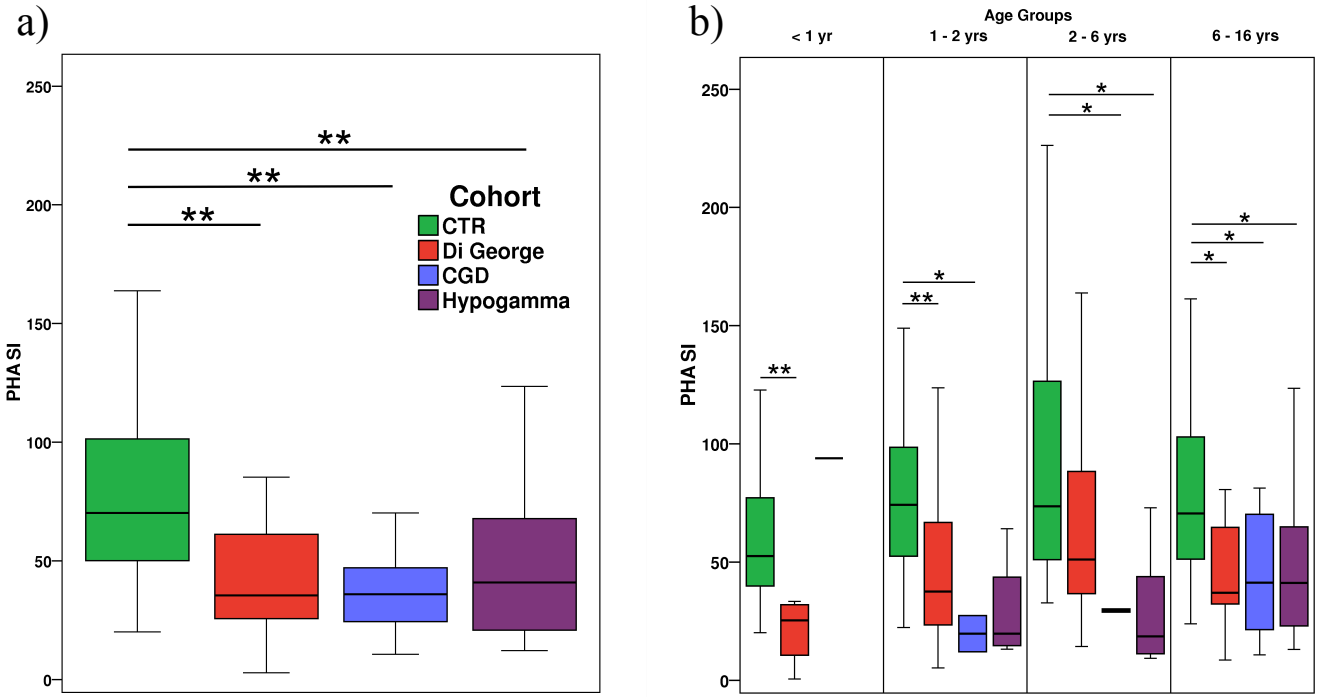


Figure 15: Lymphoproliferation in response to PHA. a) Cohort-grouped analysis, b) Age-Grouped analysis. Boxes represent the interquartile range (25^{th} and 75^{th} percentile); Horizontal bars inside boxes represent the median value; whiskers are Horizontal bars that extend from the box to the highest and lowest values, excluding outliers. Look for the number in Table 10. p-values were determined by the Mann–Whitney U-test: * 0 ($p<0.05$), ** = ($p<0.001$).

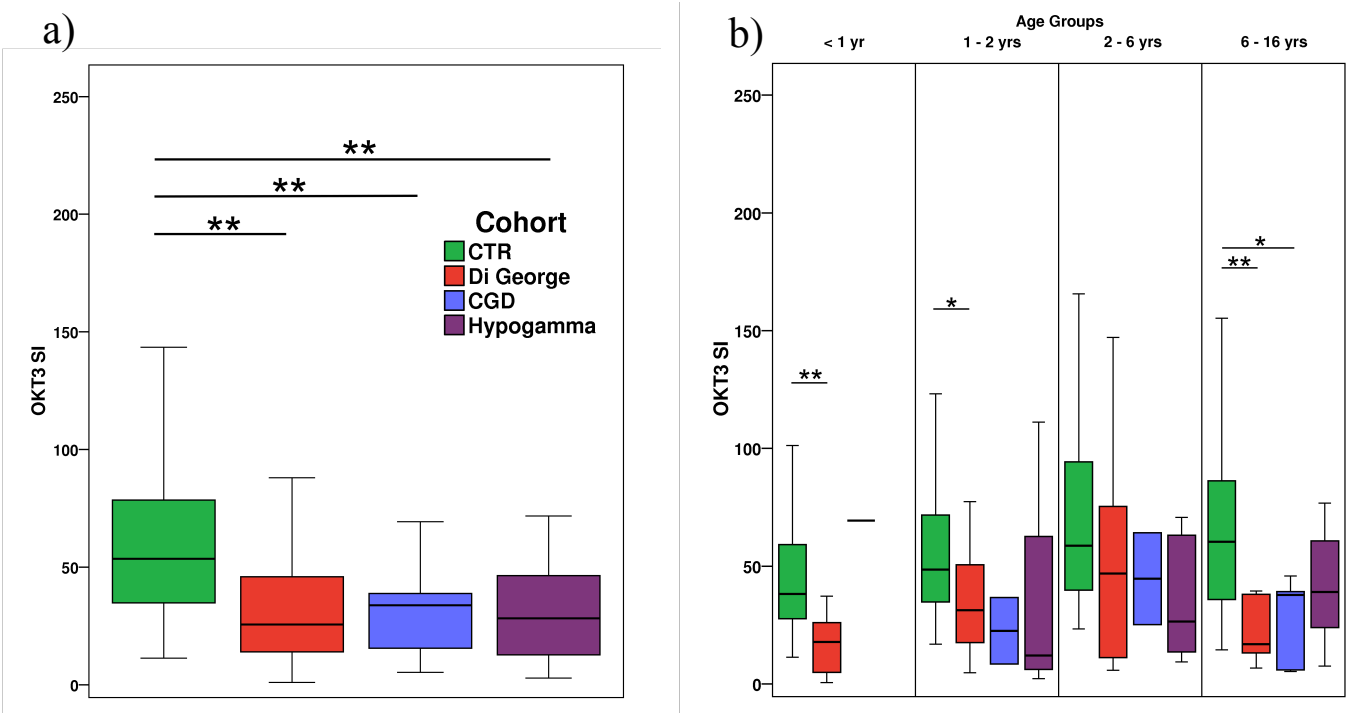


Figure 16: Lymphoproliferation in response to OKT3. a) Cohort-grouped analysis, b) Age-Grouped analysis. Boxes represent the interquartile range (25th and 75th percentile); Horizontal bars inside boxes represent the median value; whiskers are Horizontal bars that extend from the box to the highest and lowest values, excluding outliers. Look for the number in Table 11. p-values were determined by the Mann–Whitney U-test: * ($p<0.05$), ** = ($p<0.001$).

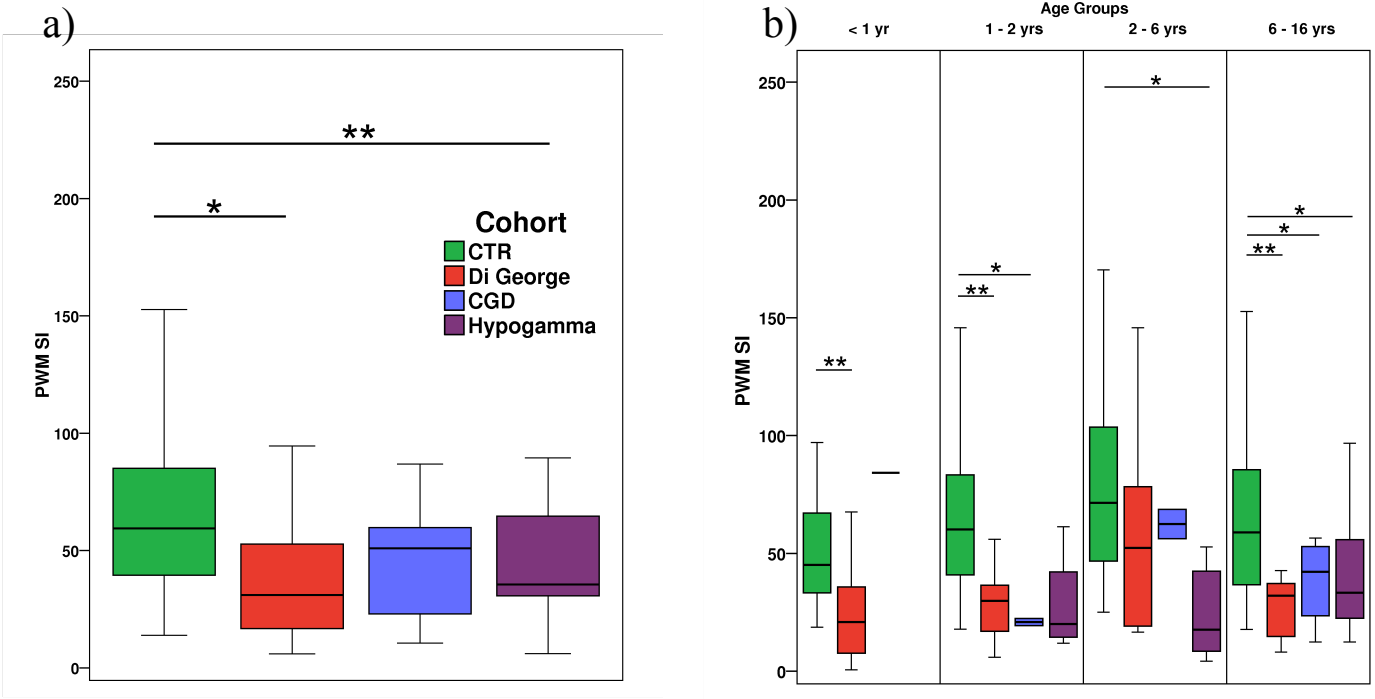


Figure 17: Lymphoproliferation in response to PWM. a) Cohort-grouped analysis, b) Age-Grouped analysis. Boxes represent the interquartile range (25th and 75th percentile); Horizontal bars inside boxes represent the median value; whiskers are Horizontal bars that extend from the box to the highest and lowest values, excluding outliers. Look for the number in Table 12. p-values were determined by the Mann–Whitney U-test: * 0 ($p<0.05$), ** = ($p<0.001$).

AKNOLEDGEMENTS

Il primo ringraziamento è rivolto al Prof. Paolo Rossi per avermi dato l'opportunità di frequentare il suo laboratorio.

Ringrazio, i miei tutor Caterina Cancrini e Andrea Finocchi per i consigli e il lavoro di revisione offerto durante la stesura della tesi. Inoltre desidero ringraziare MariaLuisa Romiti e Silvia Di Cesare, per la disponibilità e l'importante ruolo ricoperto durante la compilazione della tesi e non solo. Ringrazio Simona Piccini per la cortese collaborazione offerta per questo studio.

Un ringraziamento va a tutti i colleghi del laboratorio, in particolare al grande vicino di scrivania Roberto con il quale ho convissuto la frenesia di questa ultima lunga parte del dottorato.

Alla cara Priscilla va un profondo ringraziamento, per avermi sempre sostenuto non senza difficoltà anche durante il periodo in cui ero più assente e per avermi trasmesso un po' della sua magia. Ringrazio inoltre i miei cari amici Edoardo, Stefano e Rosalba i quali hanno cercato di farmi vedere oltre, e tutti i miei amici tra cui Andrea, Luca e Claudio per averli ancora tra questi. Desidero infine ringraziare la storica Rosanna Coen per la passione con cui è stata sempre interessata al mio percorso accademico.

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PUBBLICATIONS

Retrovirology

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Poster presentation

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Immunological response in congenital cytomegalovirus infection

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from Fourth Dominique Dormont International Conference. Host-Pathogen Interactions in Chronic Infections
Paris, France. 13-15 December 2007

Published: 9 April 2008

Retrovirology 2008, **5**(Suppl 1):P20 doi:10.1186/1742-4690-5-S1-P20

This abstract is available from: http://www.retrovirology.com/content/5/S1/P20

Background

Human cytomegalovirus (CMV) is the main cause of congenital viral infection. There are not early and certain prognostic markers to define infection /disease course and no standard treatment of children with symptomatic congenital infection is available as yet. Indeed, a small number of infants present severe neurological complications and isolated visual and hearing impairments. The aim of our study is to verify possible correlations between immunologic alterations and clinical/ therapeutic aspects. Eighteen eligible infants were enrolled in our study. Eight of them were symptomatic, showing neurological alterations.

Methods

Lymphocyte proliferation was detected by co-culture with mitogens (Phytohemagglutinin and Pokeweed), anti-CD3 monoclonal antibody, recall (Candida) and CMV-specific antigens. T-cell receptor (TCR) repertoire of CD8+ and CD4+ T-cell subsets was analysed by Spectratyping after RNA extraction and cDNA synthesis and amplification with a SuperScript One-Step RT-PCR kit (Invitrogen) by 24 different Vβ primers combination with a 3' Cβ labelled primer. IFN-γ production after CMV lisate and peptides pool stimulation was evaluated by cellELISA in 384 wells microplates.

Results

Standard immunological investigations as immunoglobulins levels and cellular immunity did not show any alteration in both groups. All symptomatic patients (8/8) did not show any specific CMV response in lymphoproliferative assay. Six out of ten asymptomatic patients showed a good CMV specific response (Stimulation Index > 3). TCR spectratyping analysis on CD8 T-cell subset showed a various degree of alteration in all symptomatic patients and in six out of nine analysed asymptomatic patients. CellELISA assay on CD4 and CD8 T-cell subset was performed and the evaluation of results is ongoing.

Conclusions

Our preliminary data suggest a possible correlation between a lack of CMV specific response and higher degree alteration of TCR spectratyping analysis in symptomatic versus asymptomatic patients.

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ABSTRACTS
SINTESI DELLE PRESENTAZIONI

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RELEVANCE OF HUMORAL IMMUNE RESPONSE IN 22Q11.2 DELETION SYNDROME.

Ariganello P., Di Cesare S., Puliafito P., Romiti M.L., Casciano F., Finocchi A., Rossi P., Cancrini C. Bambino Gesù Pediatric Hospital, Rome Italy

Background: Chromosome 22q11.2 deletion syndrome (22q11DS) is characterized by a spectrum of immunological alterations, ranging from normal profile to complete absence of T-cells ('complete' DiGeorge syndrome - DGSc). Partial DiGeorge syndrome (DGSp) shows from mild to moderate defective in T-cell, nevertheless T-cells numbers are not predictive of the risk to develop infections. Although the immunodeficiency of DGS is classically described as T- cell alterations, variable defects in the humoral compartment have been also reported.

Objective: The aim of this study is to investigate the humoral compartment in children with 22q11.2 DS.

Methods: Thirty-five 22q11.2 DS children, referred to our Center for an immunological assessment, were enrolled. Recurrent infections were defined as more than six episodes of upper respiratory tract infections per year and/or more than three lower respiratory tract infections in the previous years of life. Some patients had other associated severe infections such as septicemia, gastroenteritis and opportunistics infections. Humoral and cellular immune functions were investigated by serum immunoglobulin evaluation and testing specific antibody titers to recall antigens. Total IgE levels and autoantibodies were considered as predispositional factors to develop atopic and autoimmune manifestations. Lymphocyte subsets were assessed by flow cytometric analysis of T (CD3 CD4, CD8, CD19, CD16, CD4CD45RA, CD4 CD45Ro, CD8CD45RA, CD8CD45Ro) and B (CD19, CD27, IgD, IgM) cells subpopulations. Lymphocyte proliferation was evaluated by thymidine incorporation assay to mitogens (PHA, PWM, OKT3) and antigens (Candida and Tetanus).

Results: Eleven out of the 35 children with 22q11.2 DS enrolled had a history of recurrent and/or severe infections. Five patients received prophylactic antibiotics treatment and three of them started prophylaxis by intravenous immunoglobulin (IVIG) with a marked decreased frequency of recurrent infections. Hypogammaglobulinemia were present in 15 out of 35 (43%) patients: 7 of them had complete/partial IgA deficiency, one child had IgG deficiency, while 11 patients showed a mild decreased of the titre of IgM. Specific production of antibodies showed in 9 out of 14 patients defective response to pneumococcal polysaccharide antigen. In 3 of them the functional defect was not associated to low level of immunoglobulins.

A significant correlation between the presence of recurrent infections and humoral abnormalities ($p < 0.001$) was found.

Only in 2 out of 21 patients IgE levels were increased, both patients presented recurrent episodes of wheezing. Four out of 15 patients showed autoimmune manifestations associated to positive autoantibodies.

Total lymphocytes and CD3, CD4 and CD8 T-cells were decreased as percentage as well as absolute counts in most of DGSp patients, although these immunological abnormalities were not correlated to risk of infections. Lymphocyte proliferation in response to mitogens and antigens was normal in all patients compared to age matched healthy controls. In 15 out of 22 patients (32%) memory B-cells were matched or lower than 10th percentile. In 7 of them both subsets, memory and switched memory cells, were decreased.

Conclusion: Here we report that abnormal serum immunoglobulin levels and specific antibody deficiencies are common in patients with DGS; overall immunoglobulin abnormalities as already reported in others studies, are quite frequent in these patients and seems to be associated with the risk to developing recurrent infections. Indeed, in 55% of patients suffering of recurrent infections we observe alterations in humoral compartment, while T cell values did not correlate with infectious symptoms. An altered B cell maturation with low levels of B-cells memory was observed in the majority of patients suggesting a possible role on the impaired humoral immune response.

Obviously, antibody deficiency can contribute to the risk of infections in DGSp patients, but other factors as atopic manifestations, anatomical defects, cardiac disease, gastro esophageal reflux, and poor nutrition must be considered. In conclusion, identification of B-cell dysfunctions in DGSp could help the clinical management, with the institution of an appropriated follow-up, a more specific antimicrobial treatment and/or intravenous immunoglobulin therapy.